Ecotoxicology of Carbon Nanotubes to Sediment-Dwelling Bivalves

A thesis submitted in partial fulfilment of the requirements of Heriot-Watt University, for the award of Doctor of Philosophy
June 2020

By

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Abstract

Multi-walled (MWCNT) and single-walled (SWCNT) carbon nanotubes (CNTs) are high-aspect ratio nanostructures with a combination of properties making them useful in an increasing number of applications and products. Although CNTs occur in the environment as the result of natural combustion processes, significant environmental exposure to engineered CNTs would previously not have occurred and, therefore, they are considered to be xenobiotics of emerging concern. High concentrations of CNTs, when combined with contaminants found in sediments, are harmful to aquatic organisms and, therefore may be harmful to sediment-dwelling organisms. The present study examines the interaction of CNTs (MWCNTs and SWCNTs) with sediment, in terms of their bioavailability to the marine cockle, *Cerastoderma edule*, through *in vivo* exposure. Observations with a light microscope, confirmed by Raman spectroscopy and transmission electron microscopy (TEM), showed that MWCNTs and SWCNTs accummulated in the mantle cavity. A Raman mapping technique was also performed to detect the availability of CNTs within sediments. The present study also examined the interaction of CNTs (MWCNTs and SWCNTs) with sediment, in terms of their ecotoxicological impact on *C. edule*, with and without other sediment-associated contaminants, through three different *in vivo* exposure conditions. The MWCNTs and SWCNTs were spiked into the water column (water-spiked), spiked onto the surface of the sediment (surface-spiked) or mixed with the sediment (sediment-spiked). Their ecotoxicological impact was assessed as DNA damage using the comet assay and oxidative stress biomarkers (superoxide dismutase activity and lipid peroxidation). The bioavailability and subsequent toxicity of CNTs were governed by their interaction with sediments. It was found that CNTs mixed with sediment were less toxic than the water-spiked or surface-spiked CNTs, reflecting the feeding habits of cockles and the bioavailability of CNTs. Under the experimental exposure conditions, although SWCNTs were more toxic than MWCNTs, both CNT types were significantly toxic only at concentrations ≥ 50 μg L^{-1} [0.050 ppm] for water-spiked treatment, ≥ 100 μg L^{-1} [0.1 ppm] for surface-spiked treatment, and ≥ 0.2 μg.g^{-1} [200 ppm] for sediment-spiked treatment. In a genotoxicity assay, sediment-associated contaminants (Cd^{+2} and Zn^{+2}) were found to have a toxic effect when combined with CNTs, as they became toxic even at low concentrations of 0.1 μg.g^{-1}. Although SWCNTs were found to be more toxic to exposed cockles than MWCNTs, the DNA damage and oxidative stress in MWCNTs exposures were almost equal to those for SWCNTs when combined with sediment-associated contaminants. These results will assist the understanding of the ecotoxicology of CNTs in the marine environment, particularly in the sediment compartment, where they are expected to accumulate.

Dedication

This thesis is dedicated to

My greats parent Hassan Ashri & Nawal Arbaen
With love and eternal appreciation, there measureless support, encouragement in pursuit of excellence and success, and constant love have sustained me throughout my life..

My wonderful and lovely wife, Mona Alqassim
Who has the greatest impact in my life and with her I was able to achieve my greatest dreams..

My beloved boys, Alhassan, Ehsan and Omar
With love and being a great part of my success..

My beloved brother, Sadaqah and sisters, Afnan, Alaa and Ayat
With love and support sustained me throughout my life..

The unfortunate victims of COVID-19 and their families in 2020.

Acknowledgement

I would like to express my sincere appreciation to my supervisor, **Dr Mark Hartl**, whose encouragement, support, advice, comments throughout this study and guidance from the initial to the final level enabled me to develop an understanding of the subject.

I thank also my second supervisor, **Prof Teresa Fernandes** for her precious advice, support and guidance.

I owe my deepest gratitude to my beloved **father Hassan** and my beloved **mother Nawal** for encouraging and supporting me during my life period, many thanks again for their patience and guidance. I would like to show my gratitude to my brother and sisters for their encouragement, interest and support during my studies.

I owe my deepest gratitude to my wonderful wife **Mona Alqassim** for her support which enable me to concentrate on my research without worrying by her advices.

I am indebted to my many laboratory colleagues who supported me during my working in the lab; **Dr Majed Alshaeri, Dr Hassain Alnasheri, Dr Faisal Alqahtani, Dr Nawaf Mirza, Mr Hussam Abulkhuyour and Mr Ahmed Aljeferi.**

It is a pleasure to thank those who made this thesis possible:

Dr Mohamed Chilmeran, for his help and support in some data analysis;

Mrs Margaret Stobie, for her help and support in aquarium;

Mr Hugh Barras and **Sean McMenamy** for their support in chemical analysis;

Mr Steve Mitchel, for his support in the TEM unit at University of Edinburgh;

Mr Paul Cyphus, for his help and support in lap work;

Dr Lynn Paterson, for her advice to learn the essentials of Raman spectroscopy microscope technique;

Dr Zuzanna Gajda-Meissner, for her help in the lab.

Dr Arlene Casey, for her feedback and comments

My last (but not least), special deepest thanks to all my dear friends in particular **Mr Ahmed Almosli** and **Abdullah Alalawi** due to their encouraging me to follow independent ideas.

Research Thesis Submission (Declaration Statement)

Name:	**Naif Hassan Ashri**		
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Version: *(i.e. First, Resubmission, Final)*	**Final**	Degree Sought:	**PhD in Ecotoxicology of Carbon Nanotubes to Sediment-Dwelling Bivalves**

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E-thesis Submitted ***(mandatory for final theses)***	**E-thesis submision**									
Received in the SSC by *(name in capitals):*						Date:	**03/June/2020**			

Publications

LIST OF PAPERS

Naif H. Ashri, Mark G. J. Hartl and Teresa F Fernandes. (In revision), Ecotoxicology of Sediment-Associated Single and Multi-Walled Carbon Nanotubes in Sediment Dwelling Cockles (*Cerastoderma edule*), *The Royal Society of Chemistry; Environmental Science*: Nano.

Naif H. Ashri, Mark G. J. Hartl and Teresa F. Fernandes. (In preparation), The potential toxicity of Sediment-Associated Contaminante Cadmium and Zin with presence of Carbon Nanotubes to Sediment Dwelling Cockles (*Cerastoderma edule*) *Marine Environmental Research*.

LIST OF CONFERENCE ORAL PRESENTATIONS

Naif H. Ashri, Teresa F Fernandes and Mark G. J. Hartl. (2017). Ecotoxicology of Sediment-Associated Single and Multi-Walled Carbon Nanotube in Marine Sediment Dwelling Cockles, *NanoImpact*. Ascona, Switzerland. 17th March 2017.

Naif H. Ashri, Teresa F Fernandes and Mark G. J. Hartl. (2016). Ecotoxicology of Nanoparticles and Nanomaterials in Marine Environment, The 9th Saudi Students Conference, *The ICC*, in Birmingham, United Kingdom. 14th February 2016.

LIST OF CONFERENCE POSTERS

Naif H. Ashri, Teresa F Fernandes and Mark G. J. Hartl. (2017). The Effect of CNTs Bioavailability on Sediment Associated Contamination, E-Poster Presenter, *Marine Alliance for Science and Technology for Scotland Annual Science Meeting (MASTS).* Technology & Innovation Centre in Glasgow, United Kingdom. October 4th – 6th 2017.

Naif H. Ashri, Teresa F Fernandes and Mark G. J. Hartl. (2016). Ecotoxicology of Nanoparticles and Nanomaterials in Marine Environment, The 9th Saudi Students Conference, The ICC, in Birmingham, United Kingdom. Febrauary 13th-14th 2016.

Naif H. Ashri, Teresa F Fernandes and Mark G. J. Hartl. (2016). Ecotoxicology of Sediment-Associated Single and Multi-Walled Carbon Nanotube in Marine Sediment Dwelling Cockles, *Society of Environmental Toxicology and Chemistry (SETAC)*. Europe Annual Meeting in Nantes, France. May 22nd- 26th 2016.

Naif H. Ashri, Teresa F Fernandes and Mark G. J. Hartl. (2015). The Ecotoxicology of Sediment associated with Carbon Nanotubes, *10th International Conference on the Environmental Effects of Nanoparticles and Nanomaterials* in Vienna, Austria. Septmber 6th -10th 2015.

Naif H. Ashri, Teresa F Fernandes and Mark G. J. Hartl. (2015). The Ecotoxicology of Sediment associated with Carbon Nanotubes, E-Poster Presenter, *Marine Alliance for Science and Technology for Scotland Annual Science Meeting (MASTS).* Technology & Innovation Centre in Glasgow, United Kingdom. 30th September – 2nd October 2015

Naif H. Ashri, Teresa F Fernandes and Mark G. J. Hartl. (2015). The Ecotoxicology of Nanoparticles in marine Cockles, *3rd PhD Students Conference*. School of Life Sciences at Heriot Watt University in Edinburgh, United Kingdom. 12th March 2015

WORKSHOP

Academic Workshop: Biological and Environmental Impacts of Nanomaterials (2016). *1ST FENAC*, University of Birmingham, United Kingdom. 15th March 2016.

Acronyms

AAS	Atomic absorption spectrometry
ANOVA	One-way analysis of variance
BHT	Butylated hydroxytoluene
BMP	Breathing mode pattern
BSA	Bovine Serum Albumin
CNTs	Carbon nanotubes
CPD	critical point drying
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DSS	DNA single strand
EB	Ethidium bromide solution
EDS	Energy dispersive spectroscopy
EDTA	Ethylenediaminetetra acetic acid
ELS	Electrophoretic light scattering
ENMs	Engineered nanomaterials
FC	Flow cytomerty
HBSS	Hank's Balanced Salt Solution
LM	light microscope
LMP	Low melting Agarose
LO	Longitudinal optical
MNT	micronucleus test
MWCNTs	Multi walled carbon nanotubes
NaOH	Sodium hydroxide
NGA	Normal Gel Agarose
NMs	Nanomaterials
NPs	Nanoparticles
PBS	Phosphate buffered saline

Pi	Propidium iodide
RMB	Radial breathing mode
RMI	Residual metals impurities
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRS	Resonance Raman scattering
SCE	Sister-chromatid exchange assay
SCGE	Single-cell gel electrophoresis
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SRNOM	Suwannee river natural organic matter
SSBs	Single strand DNA breaks
SWCNTs	Single walled carbon nanotubes
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TCS	Triclosan
TEM	Transmission electron microscope
TEP	Tetraethoxypropane
TO	Transverse optical
UV	Ultraviolet

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CHAPTER 1 INTRODUCTION

1.1 Introduction

Nanomaterials have unique properties (e.g. a large specific surface area) that make them ideal for many commercial applications including consumer products in industrial and medical applications. As the demand for these products has risen in recent decades, the production of nanomaterials has also increased (Charitidis *et al*., 2014). Nanomaterials are not a new phenomenon since they are known to exist in the natural environment, with their discoveries being made by Esquivel and Murr (2004) in polar ice cores, showing that these materials had existed for more than 10,000 years. Other scholars (Luther and Rickard 2005; Navarro *et al*., 2008) reported evidence of nanomaterial creation during volcanic eruptions and in the systems of hydrothermal vents as a result of functional nuclei (e.g., nitric and sulphuric acids) simultaneous emission, and through processes like Aitken mode nucleation (Aitken 1884).

Engineered and manufactured nanomaterials (ENMs) include man-made nanoparticles (NPs) and nanomaterials (NMs) with size-dependent properties (Navarro *et al*., 2008; Frexia *et al*., 2018). This research is concerned with carbon nanotubes (CNT), one type of engineered nanomaterial. Based on the number of layers of carbon atoms, nanotubes are categorised as either single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs) (Ansón-Casaos *et al.,* 2018). Both types of Carbon nanotubes have properties that give them superior high tensile strength,

together with low mass density, high heat conductivity, a large surface area, and versatile electrical behaviours compared to those of many other high-aspect ratio materials (Upadhyayula *et al.,* 2012; Dahlben *et al.,* 2013). The production and usage of CNTs in an assortment of applications, such as electronic engineering, optics, water treatment, cosmetics and sports equipment, as well as in products in industrial and medical applications, has increased in recent decades (Bakry *et al*., 2007; Benn *et al*., 2011; Upadhyayula *et al*., 2012; Jackson *et al*., 2013; Freixa *et al*., 2018). CNTs have a potential for releasing emissions into the environment (Shvedova *et al*., 2012; Ema *et al*., 2016). However, it is unknown how these materials may impact the environment and what impact they may have on organisms, including humans (Shvedova *et al*., 2012). Accordingly, there is a pressing need for exposure levels to be examined through detailed research.

Most research to date has focused on the influence of CNTs on mammal respiratory processes (Maynard *et al*., 2004, Muller *et al*., 2006), mainly because of the need to better understand the impacts of their pathogenic properties on occupational health. There is still lack of knowledge regarding the possible environmental exposure of terrestrial and aquatic organisms to these substances. In addition, much less is known about their potential environmental impact (Kumar, 2006; Krug, 2008) and their interaction with other contaminants (Al-Shaeri *et al*., 2013). Gaining an understanding of how the toxic properties of CNTs may be propagated in marine life could provide valuable insights into their potential human impact and the transferable properties of any such chemical substances.

It is known that NMs can enter the system of lower or aquatic organisms more easily than they can enter humans, by transcytosis into the blood and lymph circulation (Oberdorster, 2006; Porter *et al.*, 2007). Some studies suggest that CNTs may cause harmful effects in these types of organisms (e.g. bivalve), by causing morphological changes in a variety of cells, and cytotoxic effects, by inducing oxidative stress and cellular toxicity, indicated by the formation of free radicals and the loss of cell viability (De Marchi *et al*., 2018). Nitrogen and oxygen free radicals are produced in living cells continuously and are considered essential to the physiological control of cell function in biological systems (Halliwell and Gutteridge, 1985).

In marine ecosystems, CNTs can remain suspended inside a body of water and then be transferred to other marine systems, such as sediments, where they might accumulate (Navarro *et al*., 2008; Freixa *et al*., 2018). As a result, sediments are predicted to become a major sink for CNT emissions, together with a number of different contaminants, including both metals and metalloids (Cross *et al*., 2015; Klaine *et al*., 2008). Thus, it is essential to understand how these nanotubes will affect organisms residing in the sediment. If CNTs are taken up by lower trophic level organisms, there is the possibility for trophic transfer, which ultimately increases the exposure to CNTs of higher trophic level organisms, which otherwise would not have been exposed to these substances. If this type of transfer occurs in commercially important species, the potential for human exposure may increase (Shvedova *et al*., 2012).

However, studying the toxicity of CNTs in terms of their chemical and biological aspects is still considered a challenge, because their surface charge, shape, size, length properties, concentration and composition can all influence the degree of agglomeration and dispersion and may impact their toxicity. This is further complicated by the significantly different approaches to CNT synthesis, the number of CNT layers and the inadequacy of testing methods using current biomarker methods (Hurt *et al*., 2006; Kennedy, 2009; Jakubek *et al*., 2009; Sahu and Casciano, 2009; Klaper, 2010; Liu *et al*., 2012; Khalid *et al*., 2016). Moreover, it has been suggested that contaminants may be bound by the CNTs, causing the former to be sequestered by them from the water column to the sediment and, as a result, possibly causing their overall bioavailability and toxicity to be decreased (Park *et al*., 2014).

While some research has been carried out investigating the behaviour and bioavailability of CNTs, as well as their ecotoxicology in the marine environment, there is still very little scientific understanding of their behaviour in sediments, where they are expected to accumulate, or the mechanisms of their interaction with organisms living within or at the surface of the sediment.

Moreover, only a limited number of studies that consider toxicity to marine organisms and bioavailability have been carried out *in vivo* to analyse CNT behaviours. The majority have focused on *Arenicola marina*, *Mytilus edulis* and algae (Templeton *et al*., 2006; Pacurari *et al*., 2008; Woods *et al*., 2009; Galloway *et al*., 2010; Long *et al*., 2012; Mwangi *et al*., 2012; Al-Shaeri *et al*., 2013; Girardello *et al*., 2015; Lukhele *et al*., 2015). Accordingly, there is a pressing need for exposure levels to be investigated

and examined, along with uptake and the fundamental impacts experienced by key organisms, including filter feeders and benthic species, particularly as any data relating to such systems is scarce.

The present study used the cockle, *Cerastoderma edule,* as a means to study the toxicity of CNTs at various minimal concentrations both in combination with metals and separately, since this species moves actively in their environment, reworking the sediment and hence modifying the chemical and physical properties of their habitat (Hedman *et al*., 2011). Moreover, as a widely acknowledged indicator of previous environmental pollution, *C. edule* has not been positioned at the fore of the majority of works on ecotoxicology, especially not when contrasted with other similar species, such as that of mussel (Al-Shaeri *et al*., 2013). Accordingly, there is a need for further study to be directed towards the *C. edule* species, particularly through examination of engineered nanomaterials (ENM) toxicity. This is vital to gather in-depth insights into any potential risks that might arise, and which could ultimately result in a negative impact on *C. edule* habitats. This would unquestionably impact the biodiversity present across such habitats. Very little quantitative ecotoxicity CNT data exist and, only recently have the possible adverse effects of CNTs on sediment-dwelling fauna become a concern (Freixa *et al*., 2018).

To the best of my knowledge, there are no reported *in vivo* studies involving CNT-induced DNA damage and oxidative stress in sediment-dwelling organisms following realistic routes of environmental exposure to CNTs recognised in various trophic layers.

1.2 Aim and Objectives

This study aims to determine the bioavailability of SWCNTs and MWCNTs to the common cockle (*C. edule*), and their possible genotoxic effects, both individually and in combination with selected sediment-associated contaminants (cadmium and zinc), using a variety of predetermined concentrations and different exposure conditions.

This research seeks to provide answers to the following questions:

1. What is the bioavailability of sediment-associated CNTs to cockles?
2. How does the toxicity of CNTs affect sediment-dwelling cockles?
3. What is the potential toxicity of sediment-associated contaminants in the presence of CNTs?

The characterisation of CNTs should be fully understood prior to starting biomarker assays, as there is a probable association between biological activity and the physicochemical properties of the CNTs (Al-Shaeri *et al*., 2013). This will define CNT interaction and behaviour with metals and other substances sediment-associated contaminants (Qiao and Aluru, 2003). These gaps will be filled by investigating several specific objectives for the three aims listed below:

1. Characterisation of stock SWCNTs and MWCNTs

Several techniques were used to assess and understand the characteristic of CNTs (SWCNTs and MWCNTs), such as TEM and Raman spectroscopy. In addition,

Dynamic Light Scattering (DLS) and zeta potential were used to assess the surface charge and the agglomeration size of both SWCNTs and MWCNTs.

2. The bioavailability of SWCNTs and MWCNTs to sediment and its resident cockles

A light microscope was used to ascertain the agglomeration/aggregation of CNTs onto cockles through their filtration system within the cockle's digestive glands and gills. Then, Raman spectroscopy was applied in order to demonstrate the CNTs' characteristics (MWCNTs and SWCNTs) to the gills of cockles *C. edule*, as well as their bioavailability within the exposed sediment. Lastly, TEM was used to establish the uptake, if any, of SWCNTs and MWCNTs by the cockles.

3. The toxicity of SWCNTs and MWCNTs to sediment-dwelling cockles in marine sediments

Comet assay was used to determine the CNTs' (MWCNTs and SWCNTs) genotoxicity towards the DNA in cockles' haemocytes and gill cells. The impact of oxidative stress caused by CNTs (MWCNTs and SWCNTs) was also investigated in relation to SOD (superoxide dismutase) and Thiobarbituric Acid Reactive Substances TBARS (lipid peroxidation) in the gills of cockles.

4. The effect of CNTs' bioavailability and toxicity of sediment-associated contamination in marine sediments

To assess the CNTs' chemical interaction with Cd^{+2} and Zn^{+2} as sediment-associated contaminants examined in this research, and to assess the uptake of these contaminants (Cd^{+2} and Zn^{+2}) by the cockle's gill filtration system, Atomic Absorption Spectrometry (AAS) was used. In addition, Trypan blue was used to investigate the effect of the interaction of CNTs (MWCNTs and SWCNTs) with dissolved metals (Cd^{+2} and Zn^{+2}) in relation to the overall viability of cells, in the case of cockles' haemocytes and gill cells. In addition, Comet assay was used to assess the genotoxicity of CNTs (MWCNTs and SWCNTs) in association with Cd^{+2} and Zn^{+2}, as sediment-associated contaminants examined in this research, and its effect on the DNA damage to cockles' haemocytes and gill cells in marine sediment. Lastly, oxidative stress was used to assess CNTs' (MWCNTs and SWCNTs) interaction with metals (Cd^{+2} and Zn^{+2}), and their effect on cockles' gills in terms of SOD and lipid peroxidation (TBARS).

1.3 Thesis Outline

Chapter 2 Literature Review: this chapter focuses on giving a general summary relating to CNTs, outlining their history, explaining what they are, their types and structure, their synthesis process, their chemical and physical properties, and, lastly, their release and corresponding environmental effects. The work and existing literature focusing on the ecological toxicity of CNTs to living organisms will be reviewed, as

well as uptake, behaviour and bioavailability with regard to their toxicity in sediments, including the influence of CNTs on the toxicity of other sediment-associated toxins. Following on, there is a discussion of the rationale behind choosing the experimental organisms examined in this work (cockles *C. edule)*. Chapter 2 also covers all CNT forms in relation to their characterisation and approaches to accumulation analysis using TEM, DLS together with zeta potential and Raman Microscopy. Subsequently, the various study approaches and biomarkers applied in this work are explained, with attention directed specifically towards Trypan blue, Comet assay, SOD assay and TBARS assay.

Chapter 3 Material and Methods: this chapter focuses on giving detailed information on the methods and biomarkers that have been utilised throughout this research. It highlights cockle collection strategies, the geographical area where the cockles have been collected from, and the aquarium that was used to house the cockles. Chapter 3 explains how TEM, DLS and Raman spectroscopy were used to characterise the CNTs. It also presents how CNT interactions with cockles and their inhabited environment were observed. This chapter also covers all the exposure conditions that were applied in relation to exploring CNT bioavailability and toxicity to cockles (*C. edule*) with and without sediment-associated contaminants. A detailed overview of cell viability (Trypan blue) method, Comet assay, SOD assay and TBARS assay are provided in this chapter. Following on, the process of determining the interaction of CNTs with sediment-associated contaminants (Cd^{+2} and Zn^{+2}) using AAS and DLS is

described. Finally, an overview of the data analysis process and the software used is given.

Chapter 4 Results: this chapter describes the outcomes of all experiments conducted in this research. It illustrates the detailed description of SWCNTs and MWCNTs' characterisation obtained by different techniques, including TEM and DLS. Then, it presents the results of all the assays, starting with CNT bioavailability to *C. edule,* followed by CNT toxicity with and without sediment-associated contaminants (Cd^{+2} and Zn^{+2}). First, the bioavailability of CNTs under the different exposure conditions was determined by histological observation, TEM examination, Raman spectroscopic mapping, is presented. Following this, the chapter presents the findings from measuring the cell viability of the haemolymph cells of *C. edule* using Trypan blue approaches. This is followed by presenting the results of genotoxicity and oxidative stress (SOD activity and lipid peroxidation) of *C. edule*. Finally, the results of the toxicity of different forms of CNTs are described and compared.

Chapter 5 Discussion: this chapter focuses on a discussion concerning the rationale for CNT characterisation and approaches to accumulation analysis using TEM, dynamic light scattering (DLS) together with Zeta Potential and Raman Microscopy. Following on, it investigates how CNTs might interact with, and be taken up by, sediment-dwelling organisms in the marine environment. This provides a better understanding of the behaviour and environmental fate of CNTs, and it is thus essential to assess their toxicity and risk for the aquatic environment. A detailed investigation on the toxicity of SWCNTs compared to MWCNTs to sediment-dwelling cockles *C.*

edule is presented. This is followed by an explanation of the various study approaches and biomarkers applied in this work, with attention directed specifically towards Trypan blue, Comet assay, SOD assay and TBARS assay.

Chapter 6 Conclusion: this chapter focuses on giving a general summary relating to CNT characterisation and bioavailability, and their toxicity alone and in the presence of sediment-associated contaminants as well as future research recommendations.

CHAPTER 2 LITERATURE REVIEW

This chapter will cover a brief history of CNTs discovery and synthesis, their structure and how they are classified. Then it discusses the types, characteristics and methods to recognise CNTs in complex media. Understanding these characteristics is important because it helps understand routes of entry of CNTs into the environment, their subsequent behaviour and transfer into living organisms. This chapter then discuss previous research and understanding of ecotoxicology of CNT and highlights knowledge gaps that this study aims to fill.

2.1 History and Development of Carbon Nanotubes

Nanotechnology is a form of molecular manufacturing, which is a branch of engineering that explores the design and manufacturing of extremely small electronic circuits and mechanical devices built at the molecular level of matter (Hahn *et al*., 2009). Although nanotechnology is a relatively recent development in scientific research, the development of its central concepts took place over a longer period. The first discovery of engineered CNTs was in 1991 by the Japanese electron microscopist Sumio IIjima at the NEC laboratory in Tsukuba, using high resolution TEM to observe carbon material (Reich *et al*., 2004). Since then, researchers worldwide have focused on investigating the structure and properties of CNTs. The first types of CNTs manufactured were multi-walled (MWCNTs). After two years, the real breakthrough was made when attempts to fill the nanotubes *in situ* with different kinds of metals led

to the development of the second type of CNTs, single-walled (SWCNTs), by Iijima and Ichihashi and Bethune and other colleagues (Bethune *et al*., 1993).

CNTs are allotropes of carbon that have a cylindrical nanostructure and are members of the fullerene structural family. A carbon nanotube is a tube-shaped material, made of carbon, having a diameter measuring on the nanometer scale, that is, one-billionth of a metre, or about one ten-thousandth of the thickness of a human hair (Reich *et al*., 2004; Frexia *et al*., 2018). CNTs have many structures, and they can differ in dimensions, including, length, width and thickness, and type of helicity (Petersen *et al*., 2011; Dahlben *et al*., 2013). Although they are formed from essentially the same graphite sheet, their electrical characteristics differ depending on these variations, acting either as metals or as semiconductors (Dresselhaus *et al*., 1995). CNTs are highly sought after for use in several different industries and applications, due to their unique properties that give them superior high tensile strength, together with low mass density, high heat conductivity, a large surface area, and versatile electrical behaviours compared to those of other high aspect ratio materials (Upadhyayula *et al*., 2012; Dahlben *et al*., 2013). Mechanically, various aspects, including the stiffness, strength and flexibility of nanotubes, are seen to be significantly higher than those of conventional carbon fibres (Salvetat *et al*., 1999; Dahlben *et al*., 2013).

2.2 Classifications and Structure of CNTs

CNTs are characterized as either single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs) (Ansón-Casaos *et al*., 2018), illustrated in Figure 2.1.

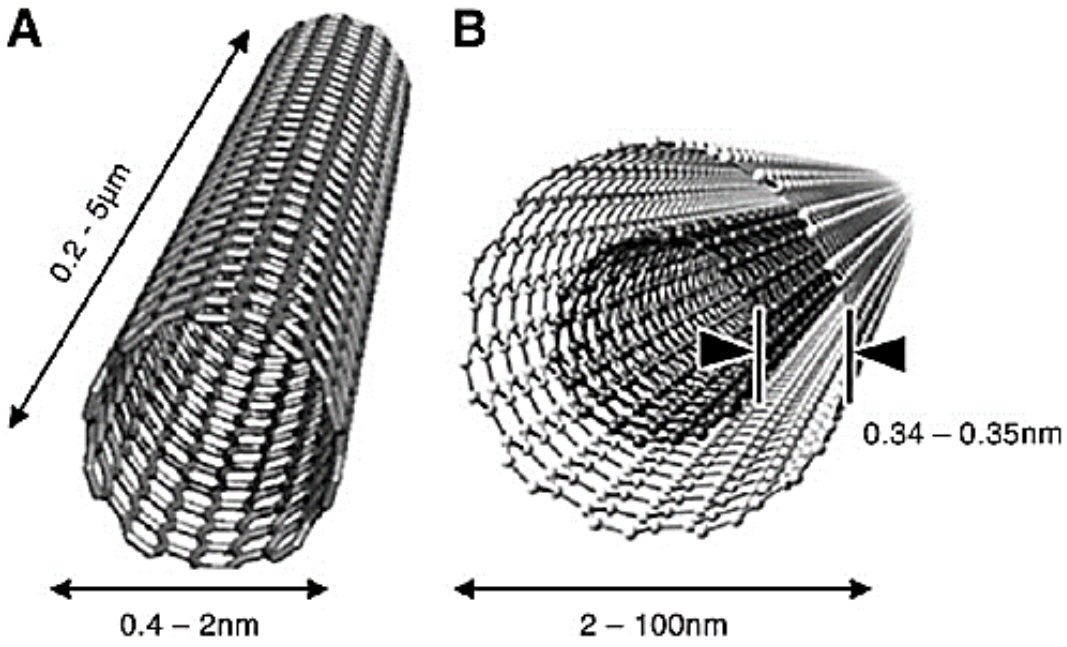


Figure 2.1: Conceptual diagram of (A) SWCNTs and (B) MWCNTs, showing typical dimensions of length, width, and inter-tube separation in multi-walled CNTs: adapted from Reilly (2007).

SWCNTs, which may be thought of as a sheet of graphene a single atomic layer thick rolled into a seamless cylinder (Petersen *et al*., 2011; Dahlben *et al*., 2013), and have a diameter range of 0.4 to 2 μm (Cheung *et al*., 2010) (Figure 2.1).

2.2.1 SWCNT

CNTs structures can be formed in three different configurations, armchair, chiral or zigzag (Dresselhaus *et al*., 1995). The configurations depend on the way the graphene is wrapped into a cylinder, represented by a pair of indices (n, m), known as the chiral vectors, while the chiral angle, *a*, can range from 0° to 30° degrees. The armchair structure, with *a* = 30°, has a metallic character. The zigzag tubes, for which the chiral angle is zero (*a* = 0°), can be either semi-metallic or semiconducting, depending on the specific diameter. A nanotube with a chiral angle in between 0° and 30° includes both semimetals and semiconductors (Dresselhaus *et al*., 1995), as shown in Figure 2.2. The integers n and m denote the number of unit vectors along two directions in the honeycomb crystal lattice of graphene. If m = 0°, the nanotubes are called the zigzag form. If n = m, the nanotubes are called the armchair form. Otherwise, they are called chiral (Dresselhaus *et al*., 1995).

SWCNTs have unique physical, electronic and optical properties (Tersoff & Ruoff, 1994) and are more pliable but harder to make than MWCNTs. They can be twisted, flattened, and bent into small circles or around sharp bends without breaking.

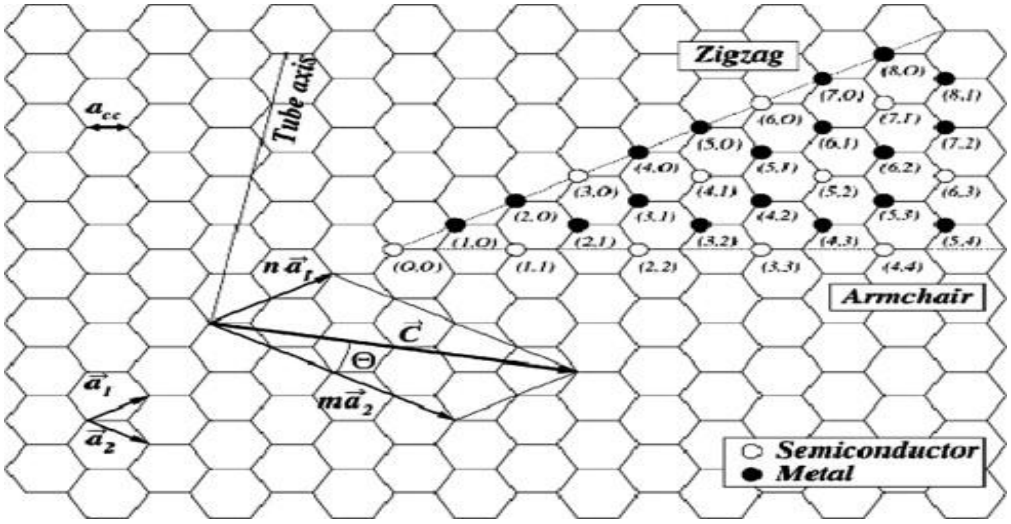


Figure 2.2: The graphene sheet diagram showing a vector structure classification used to define CNT structure. Adapted from Dresselhaus *et al*., (1995).

2.2.2 MCWNT

A MCWNT consists of multiple rolled layers of graphene that form concentric tubes (Petersen *et al*., 2011; Dahlben *et al*., 2013), and may contain between 2 to 30 concentric cylindrical layers of carbon atoms (Petersen *et al*., 2011, Sarma *et al*., 2014) with an inter-tube separation close to that of the inter-plane separation in graphite (0.34 -0.35nm) (Figure 2.1). MWCNTs have an average diameter of 1 to 3 μm for central cylindrical tubes and 2 to 100 nm for the external ones and are commonly between 20 and 50 μm dimension (Peterson *et al*., 2011; Cheung *et al*., 2010).

When it comes to providing an explanation for the overall structure of MWCNTs in line with graphite layer arrangements, two different models are used. The first model is the “Russian Doll” model, in which sheets of graphene are arranged in a structure of concentric cylinders. The other model is in the parchment-like structure where a single sheet of graphene is rolled in around itself resembling a scroll of parchment. However, the “Russian Doll” structure is more commonly produced (Allouche & Monthioux, 2005).

MWCNTs have similar properties to single-walled nanotubes; however, the outer walls on multi-walled nanotubes can protect the inner CNTs from chemical interactions with outside materials. Furthermore, it is recognized that MWCNTs offer significant tensile strength, similarly to SWCNTs.

2.3 Synthesis and Characterisation of Carbon nanotubes

Understanding the initial characterization of CNTs is essential to evaluate the behaviour and thus the possible toxicity of SWCNTs and MWCNTs and their interaction with other substances in a biological system. Table 2.1 shows parameter properties and their relative importance for the characterization of CNTs for toxicity studies (Sahu and Casciano, 2009).

Table 2.1: Some of the most fundamental aspects of nanomaterial characterization in the case of work related to toxicity (Sahu and Casciano, 2009).

Property	Importance for toxicity testing	Comments
Particle size distribution	Essential	
Degree/state of agglomeration	Important	
Particle shape/shape distribution	Important	
Chemical composition/purity	Essential	
Solubility	Essential (where applicable)	
Surface properties. Specific surface area/porosity	Essential	Surface roughness may be important
Surface chemistry/reactivity	Essential	In some cases, may be the mechanism of toxicity
Surface adsorbed species	Important	
Surface charge/Zeta potential	Important (essential under aqueous conditions)	Especially in aqueous biological environment, may change according to the environment
Physical properties	Important	
Density	If applicable	
Crystallinity	If applicable	
Microstructure	If applicable	
Optical and electronic properties	If applicable	
Bulk powder properties	If applicable	May be important for dosimetry/exposure
Concentration	Essential	Can be measured as mass, surface area, or number of particles.

The term nanomaterials (NMs) has been generally agreed to describe any material whose physicochemical structure is on a greater scale than commonplace molecular dimensions, but which is nonetheless less than 100nm in at least one dimension (nanostructure) (Klaine *et al*., 2008; Ema *et al*., 2016). According to the definition of the European Commission (2011): "Nanomaterials are materials whose main constituents have a dimension of between 1 and 100 billionth of a metre." When new man-made substances are introduced into the environment there is always concern that they may be toxic or environmentally damaging. Studying the potential toxicity of CNTs, as any other NMs, in terms of their chemical and biological aspects is still considered a challenge, because their surface charge, shape, size, length, properties, composition and concentration can all influence their agglomeration, dispersion, along with inadequate or insufficiently uniform testing methods, may impact their toxicity (Figure 2.3). Agglomeration is an assembly of particles which joined together at the edges or corners, while the aggregation whose total surface area does not differ perceivable from the sum of specific surface areas of particles. Agglomerate is the term that has bee used throughout this thesis.

Characterizing NMs in specific media, prior to assessing toxicity, both *in vivo* and *in vitro*, is fundamental, with several critical factors recognized as affecting toxicity, including particle composition, reactivity in the media, shape, size, distribution, and the surface area and chemistry (Murdock *et al*., 2008). Physico-chemical aspects, including surface charge, are recognized as playing an important role in SWCNTs toxicity (Al-Shaeri *et al*., 2013). Importantly, the effects of size have been analysed in

the study by Hund-Rinke and Simon (2006), who observed that 25nm TiO_2 particles were toxic to the green algae *Desmodesmus subspicatus* (EC50 44 mgL^{-1}), whilst 100nm TiO_2 particles were not. Furthermore, it was explained in the study by Helland *et al*. (2008) that a larger CNT surface area can re-position other molecules by picking them up and moving them across the environment. Poland *et al*. (2008) have suggested that there may be a number of different asbestos-type toxicities linked with CNTs. The physicochemical aspects of CNTs may also be more reliant on the dimensions of the nanotubes, as well as their overall bundle-forming behaviour (Badaire *et al*., 2004). This is also complicated by the varying approaches by which they are synthesised, in addition to the number of layers (Maynard *et al*., 2004; Hurt *et al*., 2006; Kennedy, 2009; Jakubek *et al*., 2009; Sahu and Casciano, 2009; Klaper et al., 2010; Liu *et al*., 2013; Khalid *et al*., 2016; Freixa *et al*., 2018; Lead *et al*. 2018).

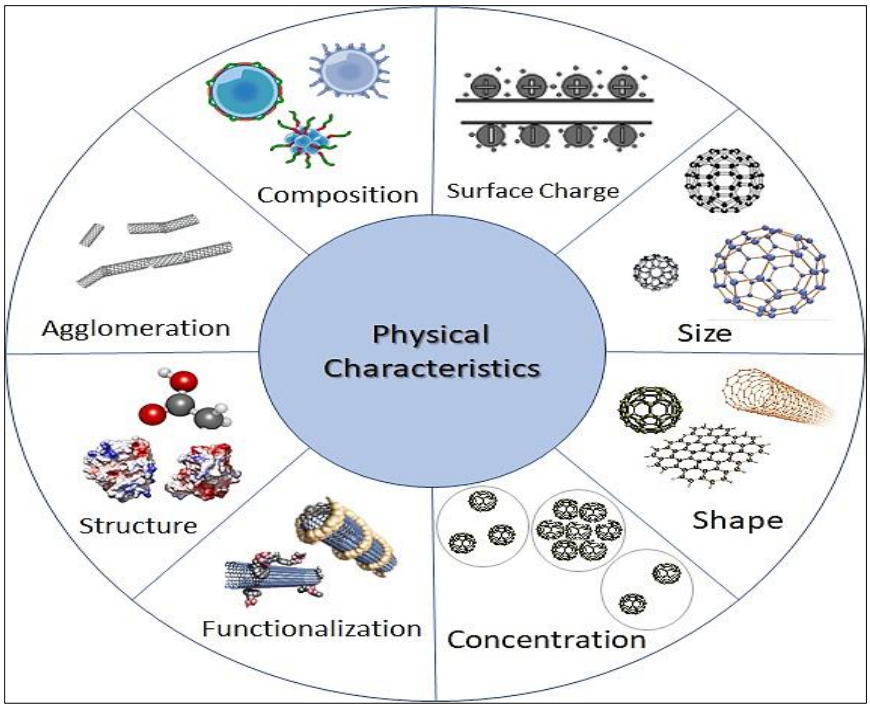


Figure 2.3: Key physicochemical properties of CNTs

In the case of NMs, it has been established that some ENMs are not toxic at expected real environmental concentrations on aquatic organisms (Frexia *et al.,* 2018). For example, concentration of some NMs such as C_{60} in the range of μgL^{-1}, where not found to be toxic in wastewater (Bäuerlein *et al.,* 2013), however, at higher concentrations, in the range of mg L^{-1} C_{60} toxic effects in the green alga *Scenedesmus obliquus* have been observed (Tao *et al.,* 2015). However, there are different variables affecting the toxic concentrations, such as exposure time, ENMs preparation methods and the type of aquatic organism (Frexia *et al.,* 2018). For example, carbon nano materials (CNMs) are considered to be non-toxic for bacteria, while they are slightly toxic for algae, crustaceans, and affect fish to a lesser extent (Sohn *et al*., 2015). Also, NMs dispersed using organic solvents have shown clearly higher levels of toxicity than mechanical methods (Henry *et al.,* 2007). Moreover, the toxic effect of conventional contaminants in aquatic environments could increase in the presence of CNMs (Frexia *et al.,* 2018). Therefore, because there are all these different factors to be considered, there is a need for many different types of studies to investigate the toxicity at various scales.

The CNTs synthesis processes **(See section 2.3.1)** include thermal vaporization of metal catalysts, carbon and different manufactures that employ different carbon sources and catalytic metals, to yield raw CNTs of varying length and purity, which are two important characteristics to determine the potential health risk and the behaviour of CNTs following exposure (Lam *et al*., 2006).

2.3.1 Methods of Synthesis of CNT

Arc discharge, laser ablation and Chemical Vapour Deposition (CVD) (Dahlben *et al*., 2013) are among the main techniques used to produce CNTs. The group led by Richard Smalley from Rice University (Houston, TX) refer to CVD as the HiPco process (Lam *et al*., 2006). However, although there are several techniques that make it possible to grow the nanotubes, these are less successful, because of the price of the catalyst material, the expensive reaction apparatus and the complex reaction conditions, e.g., achieving the required temperatures of liquid nitrogen at high pressure. As a result, the former synthesis processes have been adapted and improved to enable new conditions to be used for the process rather than to discover new technologies. Currently, arc discharge and CVD are applied widely to form carbon nanotubes (Szabó *et al.,* 2010).

2.3.1.1 Arc Discharge

The initial discovery of synthetic CNTs was by using the arc discharge technique, which has been the most commonly used method to synthesise CNTs. In 1991, during an arc discharge which was intended to produce fullerenes, CNTs were observed by using a current of 100 amps (Iijima, 1991). In 1992 the first macroscopic CNTs were presented, as a result of the work of two academics at the Fundamental Research Laboratory of NEC (Ebbesen and Ajayan, 1992). This used arc discharge, where the high temperatures result in sublimation of the carbon held in the negative electrode. In this method, a vapour is created by an arc discharge between two carbon electrodes

with or without a catalyst. Importantly, as a result of the carbon vapour being produced, the self-assembly of CNTs is witnessed.

2.3.1.2 Laser Ablation

When implementing the method of laser ablation, a high-power laser beam impinges upon a particular volume of feedstock gas containing carbon, as in the cases of carbon monoxide and methane material (Aqel *et al.*, 2013). For example, laser ablation is more successful in producing small quantities of pure CNTs when compared to arc discharge approaches, which are known to generate significant volumes of impure material (Aqel *et al.*, 2013). Further examples include Smalley group's adaptation of the approach applied in order to create fullerenes; this was proven to be effective in relation to SWCNT synthesis, achieving a greater yield and improved levels of purity when contrasted with the arc process (Arepalli *et al*., 2004).

2.3.1.3 Chemical Vapour Deposition (CVD)

The most widespread method currently used to produce CNTs is chemical vapour deposition (CVD), owing to the fact that it is simple to up-scale, which favours production in the commercial domain (Daenen *et al*., 2003). However, this technique only produces MWCNTs and poor quality SWCNTs (Aqel *et al*., 2013).

Additionally, there are other synthetic processes to produce CNTs, such as electrolysis (Hsu *et al*., 1995), and the approach which achieves CO disproportionation through high pressure (HiPco) (Nikolaev *et al*., 1999; Bronikowski *et al*., 2001), the plasma

torch method (Alford *et al*., 2001), the flame synthesis method and solar energy, which has been proposed, particularly for the synthesis of SWCNTs (Aqel *et al*., 2013).

Currently, CNTs are sold through the Aldrich Chemical Company Inc., amongst others, where CNTs are made through the HiPco or laser process. The raw CNTs, produced using the HiPco process, are available from Rice (Figure 2.4) (Lam *et al*., 2006).

Table 2.2: Synthesis Processes of CNTs, including metal content (Kumar *et al*., 2006).

Test Material	Manufacture	Synthesis Process	Metal Content (%)
Soot containing CNTs	Toyo Tanso Co. Ltd., Japan	Electric arc	Co/Ni No info on %
SWCNTs	Rice University, Houston, TX	Laser	Ni: 10%
SWCNTs	Rice University, Houston, TX	HiPco	Fe: 26.9% Mo: 0.95% Ni: 0.8%
SWCNTs, purified	Rice University, Houston, TX	HiPco	Fe: 2.1%
SWCNTs	CarboLex Inc. Lexington, KY	Electric arc	Ni: 26.0% Y: 5.0% Fe: 0.5%
SWCNTs	DuPont Co. Wilmington, DE (or Rice University, TX)	Laser	Ni: 5% Co: 5%
SWCNTs, purified	Carbon Nano-Technologies Inc., Houston. TX	HiPco	Fe: 0.23%
MWCNTs	Facultes Universities Notre-Dame de la Paix, Namur, Belgium	CVD	Co: 0.95% Fe: ~1%

To the naked eye, both types of CNTs look like a black powder and share a number of common features. After the removal of most of the undesirable residual metallic impurities (RMI) (Mn, Ni, Cu, and Fe), CNTs are sold in ‘purified’ form. Although nitric acid can extract RMIs from CNTs, it can also produce carbonaceous impurities

when they react with CNTs, where the walls of CNTs can be broken; however, the amount of carbonaceous impurities and the degree of damage can be regulated during the synthesis process (Chin *et al*., 2007). Furthermore, when considering the most widely applied gaseous carbon sources, acetylene, carbon monoxide, ethanol, ethylene and methane are highlighted (Moisala *et al*., 2003). Although it is possible to produce MWCNTs without the presence of metal, the presence of even a small amount of the metal catalyst helps to align the nanotubes (Lam *et al*., 2006). If graphite is the material of both electrodes (in other words, pure graphite electrodes) the main product will be MWCNTs; however, in order to produce SWCNTs, the anode needs to be doped with a metal catalyst, including, for example, Co, Fe, Y, Mo, or Ni (using a mixture of metal catalysts with graphite) (Table 2.2).

SWCNTs and MWCNTs are known to have van der Waals forces acting between their molecules, which means they are forced to combine to form microscopic bundles or ropes; subsequently, these bundles transform to become small clumps. However, the van der Waals forces in MWCNTs are recognized as being not as effective as those of SWCNTs. Owing to this feature, MWCNTs adopt the form of individual tubes, with only a small number of bundles created, comparable to microscopic ropes (Figure 2.4 F and G). Nevertheless, the appearance of bulk MWCNTs may be described as being comparable to SWCNTs, as can be seen in the diagram below. Generally, MWCNTs are more difficult to study and characterize and more heterogeneous compared to SWCNTs (Lam *et al*., 2006).

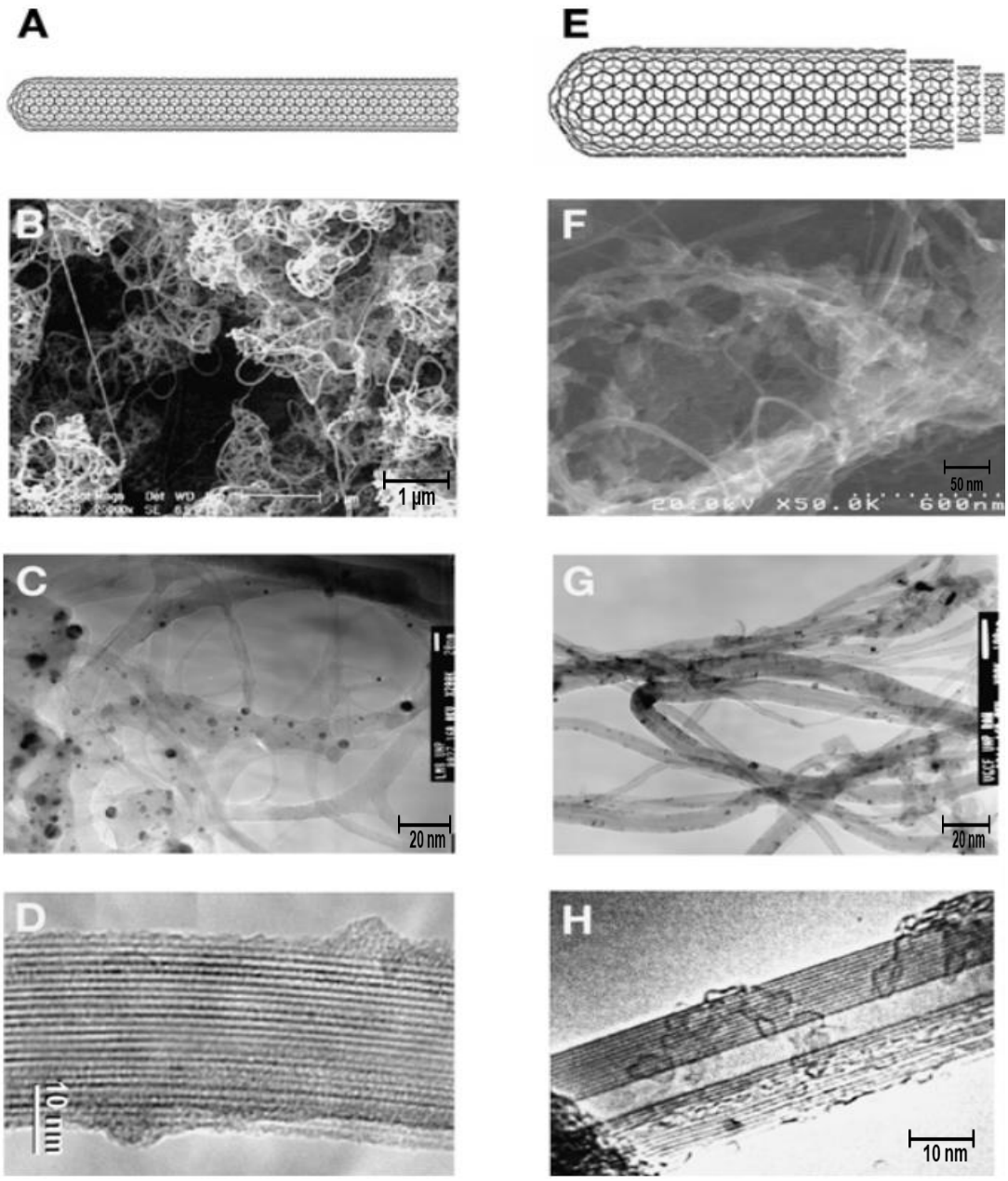


Figure 2.4: (A) to (D): SWCNTs; (E) to (H): MWCNTs. SEM pictures provide an overview of the aggregates of SWCNT (B) and MWCNT (F); the TEM images depict raw SWCNT ropes with the addition of metal nanoparticles (C), as well as the presence of individual multiwalled tubes (G). The high resolution TEM figures provide a cross-section of an SWCNT bundle (D), which is made up of >25 tubes, in addition to various instances of amorphous carbon, as identified across the edges, as well as a longitudinal cross-section of a MWCNT (H); the central cavity is empty, and each side is seen to encompass ~20 walls, in addition to amorphous carbon (Nikolaev, 1999; Lam, 2006).

2.3.2 Characteristics, Methods of Detection

According to Kingston (2007), applications of CNTs need to be tailored to their function; hence, it is critical to characterise CNTs when determining the volumes required, in addition to the desirable properties and qualities. Current techniques to characterize CNTs include photoluminescence spectroscopy, electron microscopy (TEM & SEM), x-ray photoelectron spectroscopy (XPS), x-ray diffraction (XRD), absorption spectroscopy (UV-Vis and IR), thermal analysis (TGA/DTG), neutron diffraction and Raman spectroscopy. This section discusses the main method for detecting CNT, which is Raman spectroscopy, and the main method for determining the size of CNTs, which is Dynamic Light scattering (DLS).

2.3.2.1 Raman spectroscopy

Raman spectroscopy is widely acknowledged as a valuable approach to obtaining insights into the characteristics of carbon-based materials, including CNTs and carbon black (CB), and is commonly recognized as a fundamental instrument when seeking to obtain insights into a number of critical elements of all sp2 carbons (Bandow *et al*., 1998; Popov *et al*., 2000; Dresselhaus *et al*., 2010). Raman spectroscopy has been applied to measure the diameter of nanotubes (Graupner, 2007), and measure the degree of disorder in sp2-hybridized carbon systems (Cuesta *et al*., 1994), as well as the effects of nanotube-nanotube interaction (Rao *et al*., 2001) on the vibrational modes. A number of particular aspects have been the focus of several in-depth studies, including the strong frequency dependence on the excitation laser energy of specific

Raman bands (Matthews *et al*., 1999; Thomsen and Reich, 2000; Brown *et al*., 2001; Sood *et al*., 2001), or other laser radiation-induced influences (Li *et al*., 2000; Zhang *et al*., 2007). The Raman spectroscopy resonance supply an essential instrument for biological detection (Alivisatos, 2004). For example, SWCNTs has been detected in mice's kidney, bladder and intestine (Liu *et al*., 2008), and in the gill of mussels *Mytilus edilus* (Al-Shaeri *et al*., 2013) and gill of *Daphnia magna* (Petersen *et al*., 2013).

The Raman spectra of CNTs are commonly seen to demonstrate at least three characteristic bands, namely the D mode (~1350 cm^{-1}), the tangential stretching G mode (1500–1600 cm^{-1}), and the radial breathing modes (RBMs) (100–400 cm^{-1}) (Rao *et al*., 1997; Jorio *et al*., 2004; Dresselhaus *et al*., 2005). In the majority of instances, however, MWCNTs do not produce characteristic RBM Raman signals (Dresselhaus *et al*., 2005). Essentially, the position of the G band is recognized as being relatively similar when compared to that of graphite (~1580 cm^{-1}).

When examining SWCNTs, the preliminary characteristic signature is that of G-bands, which are thought to originate as a result of two adjacent carbon atoms being identified as present on the walls of the SWCNTs, together with their optical vibration, which are seen to have a smaller G^- peak whilst showing a stronger G^+ peak (Figure 2.5). The latter can be recognized through the LO (longitudinal optical) approach, with the link identifiable when examining the nanotube's vibrations. In contrast, the weak G^- peak seems to demonstrate a lower frequency, which is recognizable through the transverse optical (TO) mode, with its link apparent through circumferential direction

vibrations. A low volume of low-frequency phonon mode normal to the graphene sheet is added on the comparative (ω = Ω) down-shift connecting ωG and ωG+ (Iliev *et al*., 2000).

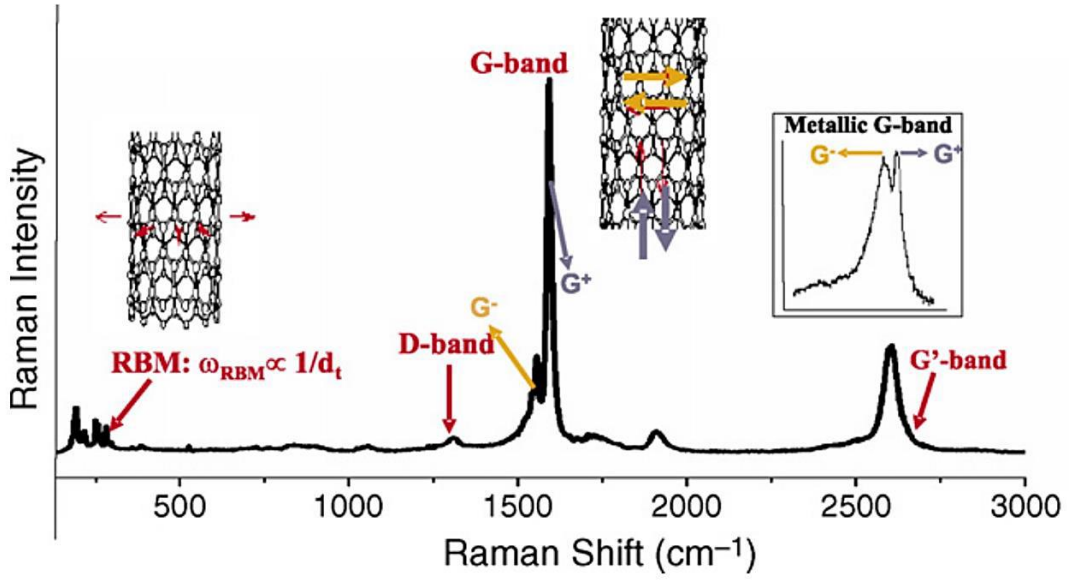


Figure 2.5: Raman Spectroscopy for SWCNTs (Dresselhaus *et al*., 2007).

In the case of metallic tubes, there is a link between the G^+ mode and that of the LO mode and the TO mode, where the link is seen to demonstrate a dip when there is an instance of lower frequency, as highlighted by various researchers (Baughman *et al*., 1999; Bandow *et al*., 1985; Dresselhaus *et al*., 2007); This is considered to be owing to the significance in interaction with the electron phonon. Importantly, when examining a Raman spectrum, the intensity considered as being high frequency is 1500–1600 cm^{-1}, whereas the range determining low frequency is recognized as 140-250 cm^{-1}, whereas in the case of RMB (Radial Breathing Mode), the zone centre in-

phase may be seen to be inversely proportional to the tube's radius and is independent (Iliev *et al*., 2000; Fujimori *et al*., 2013).

In a SWCNT, the second most important characteristic signature is the presence of a strong Raman aspect across predominantly low frequencies, indicating a link with RBM, with the vibration of all carbon atoms in the radial direction in an asymmetry, totally symmetric, breathing mode pattern (BMP), unique to SWCNTs. According to Yowell *et al*. (2002), ωRBM frequency vibration is recognized alongside $1/d_t$ dm, which therefore provides an approach to characterization in regard to establishing the distribution of SWCNTs diameters. The key reliance on laser excitation energy, E_{laser}, demonstrated by these two individual characteristic Raman features is interpreted in terms of a resonance Raman scattering (RRS) approach, as identified by Leite and Porto in their work at the US-based Bell Labs (Nikolaev *et al*., 1999).

When examining the Raman spectrum of the MWCNTs' bundles, two key graphite bands can be identified, namely the band at 1342 cm^{-1} (D band), which is seen to be induced through the identification of disorder, in the case of carbon systems, and then the band at 1580 cm^{-1} (G band), which is allocated to the C–C bond's in-plane vibration (G band), where the shoulder is recognized as being in the region of approximately 1604 cm^{-1}, which is characteristic of imperfect or otherwise flawed graphite-like materials (Figure 2.6). Furthermore, a band at 2683 cm^{-1} is also identifiable in the Raman spectrum, and is referred to as the G′ band and ascribed to the D band's overtone. Importantly, all of the bands mentioned are identified and confirmed on the bundled MWCNTs' powder as-received.

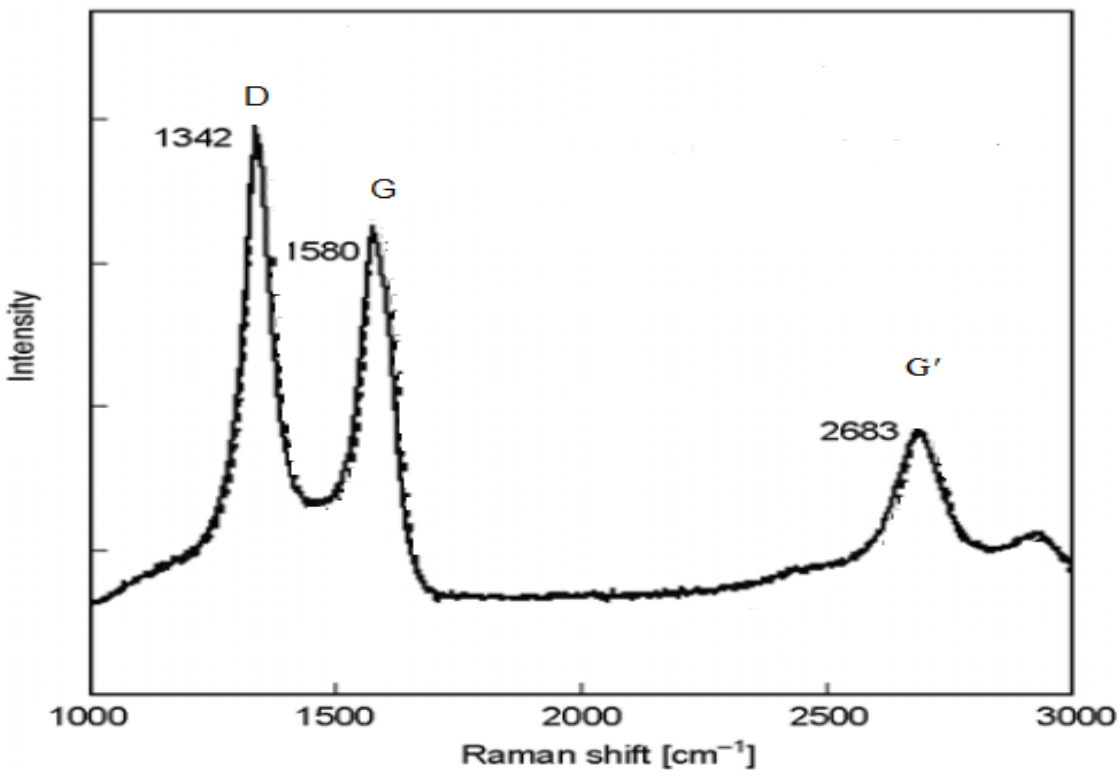


Figure 2.6: Raman Spectroscopy for MWCNTs. Adapted from Fernando *et al*. (2013).

In contrast to SWCNTs, another Raman band is exhibited by MWCNTs, most distinctively at ~1615 cm^{-1}, and is referred to as the D' band. Comparable to the D band, this particular band is recognized as being a double-resonance Raman characteristic, stemming from defects, disorder or otherwise through ion intercalation in the graphitic walls (Solin and Caswell; 1981; Dresselhaus and Dresselhaus, 1982; Rao *et al*., 1997; Jorio *et al*., 2003; Dresselhaus *et al*., 2005).

2.3.2.2 Dynamic Light Scattering (DLS)

Powers *et al*. (2006) reviewed various approaches to nanomaterial characterization and particularly recommended DLS as a valuable approach when seeking to evaluate various aspects in regard to NMS, including the size of particles, their size distribution, and also the zeta potential (±), in the case of dispersions. Going as far back as 1975, DLS has been applied as a simple approach to measure the size of particles in a dispersed, along with a corresponding examination of their capacity for suspension. Measuring the frequencies of small particle size in gases, solids and liquids is no longer as complicated, as a result of developing laser technology, as highlighted in various works (Berne, 1976; Simakov and Tsur, 2007; Wu *et al*., 2005; Pecora, 2013). DLS can be used to estimate and determine the particle size of Engineered Nano Materials (ENMs) as mentioned by Rocha (Rocha *et al*., 2015). The hydrodynamic diameter (dh) can be defined as the size of the ENMs and the agglomerates, which depends on the size of the particle. In addition, the DLS can measure the Brownian movement of NMs suspended in the liquid (Pelley and Tufenkji, 2008). The DLS can be also provide the hydrodynamic diameter of the CNTs agglomerates from which the approximate size of these agglomerates can be inferred (Smith *et al*., 2008; Al-Shaeri *et al*., 2013), which can be used to estimate the various length distributions of the CNTs (Cheng *et al*., 2011). DLS is utilized to determine the approximate diameter of CNT agglomerates in suspension, whereas Electrophoretic Light Scattering (ELS) generates the zeta potential that provides data regarding the degree of dispersion (Sano *et al*., 2001; Gao *et al*., 2003; Lee *et al*., 2005).

The equation provided below is the most commonly applied when examining particle size.

Equation 2.1

$$D_h = \frac{K_B T}{3\pi\eta D_t}$$

D_h is the hydrodynamic diameter.
D_t is the translational diffusion coefficient.
k_B is Boltzmann's constant.
T is thermodynamic temperature.
η is dynamic viscosity.

2.4 Entry of NMs into the Marine Environment

The constantly increasing number of CNTs in consumer products (Upadhyayula *et al*., 2011), aerospace (Baur and Silverman, 2007), construction, medical (Armentano *et al*., 2010; Sahithi *et al*., 2010) and other industrial applications of CNTs on a worldwide scale means that their potential for release into the environment is likely to increase. Evaluating their potential risk to the environment requires an understanding of their environmental fate, bioavailability and toxicity (Krug, 2008) (Figure 2.7).

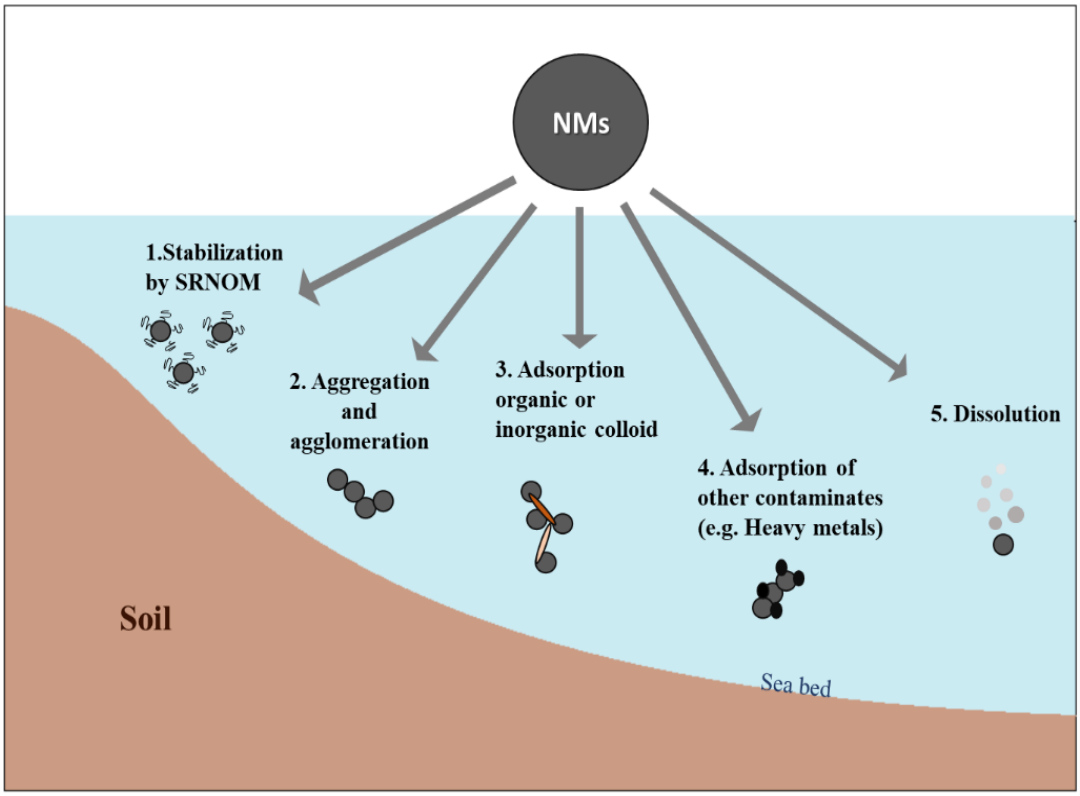


Figure 2.7: The Potential Behaviour of NMs in Aquatic Environments.
NMs = nanomaterials. SRNOM = Suwannee River Natural Organic Matter.

When examining potential CNT toxicity, two fundamental consideration to be taken into account is the source of CNTs and their entrance route in to the marine system. CNT release scenarios are defined by Nowack *et al*. (2013) as the environmental or operational circumstances that lead to the release of CNTs. Therefore, CNT composite materials are being released into environments across all their different lifecycle stages. These environments include homes and workplaces, for example, and incorporate the different parameters used to explain the form, type and scale of release.

CNT release could occur during any stage of their lifecycle, from the production of CNTs, through the manufacturing process, to their use and end-of-life disposal/ management (Curran, 1996; Upadhyayula *et al*., 2011; Upadhyayula *et al*., 2012; Nowack *et al*., 2013). CNTs' entrance pathways into marine systems include direct input, such as through water effluents and sewage, and indirect input, such as atmospheric depositions (Nowack and Bucheli, 2007; Muellere and Nowack, 2008; Freixa *et al*., 2018).

Upon release of CNTs into the environment, in the ways mentioned above, it is possible that abiotic interactions will be identified, depending on the conditions present in different atmospheric, aquatic and terrestrial environments, subsequently resulting in changes of physical and chemical perspectives. Such changes are fundamental when it comes to establishing the fate of CNTs in the respective setting and, as a result, their availability to organisms. If there is close proximity between organisms and CNTs, biological interactions will occur, causing CNTs to enter such organisms. Once the CNTs are inside the organisms, the toxicokinetics involved will

result in different outcomes, with trophic transfer via food webs, and the subsequent toxicodynamics potentially impacting the ecosystem as a whole and affecting communities (Maynard *et al.,* 2004; Nowack and Bucheli, 2007; Muellere and Nowack, 2008).

The environmental behaviour and the effect of CNTs in an aquatic system are associated with various factors that may increase the chance of CNT mobility within the environmental compartments. Firstly, the mobility of CNTs is influenced by the chemical properties of the CNT surface that in turn are determined by the characteristics of the medium, e.g. pH and ionic strength; these conditions facilitate their mobility. Secondly, CNTs can be very stable throughout their environmental transportation pathway, because of their high resistance to biodegradation; thus, their chance of passing through different compartments in the environment are increased. Thirdly, in various applications, CNTs can be designed to increase their dispersion and stability in aqueous media, and such modifications may also enhance their mobility in the environment (Mattison *et al*., 2011; Handy *et al*., 2012; Bundschuh *et al*., 2016; Freixa *et al*., 2018).

As a result of the greater degree of agricultural and industrial activities, as well as a significant increase in urbanization, pollution has been recognised as a key threat to aquatic systems. In this respect, it is widely acknowledged that sediments are a sink for a number of contaminants, such as metals, metalloids and other inorganic and organic substances. This particularly affects benthic organisms, which may live close

to or within the sediment such as cockles (*C. edule*) (Quintino *et al*., 2006; Elliott and Quintino, 2007; Langston *et al*., 2010).

The increased commercialisation of CNTs raises the issue of not only the possibility of their release to the environment but also the fundamental concern that organisms may face exposure and a toxic response (Mueller and Nowack, 2008), including humans (Gorman, 2002). Disposing of CNTs after their production and use will lead to their presence in environment, where they can be taken up by aquatic organisms, which has been found to potentially lead to cell damage (Wiesner *et al*., 2006; Petosa *et al*., 2010). CNTs are likely to accumulate in sediment, where marine organisms, such as cockles (*C. edule*) can take them up.

Sediment is an essential and natural component of ecosystems, which provide substrate and habitat for different organisms. It also plays vital roles in many fundamental ecosystem services and functions as well as a key component in biogeochemical and hydrological cycles (Wall, 2004). As a dynamic component of the hydrologic cycle which links terrestrial, freshwater and marine ecosystems, sediment also plays a role in a wide range of human activities within aquatic systems, including water resource maintenance, flood protection, the maintenance of navigability ecosystem protection, and the protection of coastlines (Apitz *et al.,* 2012). In addition, sediment supply to fluvial systems is affected by a range of landscape management approaches (Apitz *et al.,* 2012). Sediments play an important role in aquatic ecosystems, as they serve as both a sink and a source of organic and inorganic materials. Sediments are the ultimate recipients of CNTs released to the environment

and this can alter the sediment's microbial activity (Chung *et al*., 2011). However, there are many negative effects of sediment contamination on an ecosystem, some of which can be clearly observed and others which are more hidden (Hoffman *et al*., 2002). One of these is that benthic invertebrate communities can be totally lost or converted from sensitive to pollution-tolerant species (Hoffman *et al*., 2002). Environmental impact is assessed by measuring the various amounts and effects of specific contaminants in different organisms. In environments involving sediments and water, emphasis has been placed on observing and examining benthic community parameters, including the species present, their abundance and, evaluating any toxicity that may have been induced in these organisms (Connon *et al*., 2012).

2.5 The Ecological Toxicity of CNTs

The unique properties inherent in ENMs have given rise to much controversy, in terms of widely considered hypotheses and insights into ecotoxicology, which has resulted in nanoecotoxicology being recognized as a subgroup of ecotoxicology (Kahru and Dubourguier, 2010).

The controversial and sometimes inconsistent findings (Buzea *et al*., 2007; Gonzalez *et al*., 2008) from *in vivo* CNT toxicity studies, particularly in relation to aquatic organisms, are associated with various relevant factors, such as the type of CNTs, exposure time, the type of aquatic organisms and the variety of methods used in preparing the CNTs (Freixa *et al*., 2018), as well as the dispersants used (Oberdörster, 2004; Handy *et al*., 2008b).

Carbon nanotubes can be functionalised to attain desired properties that can be used in various applications. The resulting nanotube aggregates or bundles reduce the mechanical performance of the final composite. The surface of the carbon nanotubes can be functionlised (modified) to reduce the hydrophobicity and improve interfacial adhesion to a bulk polymer through chemical attachment (Stando *et al.*, 2019). Several studies have concluded that different types of CNTs showed significant differences in their ecotoxicity, which may be attributable to their different aggregation behaviour, surface functionalization, chemical composition, and preparation methods (Jackson *et al.*, 2013). For example, although only non-toxic effects were reported on different groups of animals exposed to functionalized CNTs (Dumortier *et al*., 2006; Wu *et al*., 2008), a profound toxic effect was observed on mice lungs which had been exposed to non-functionalized CNTs in *in vivo* studies (Lam *et al*., 2004; Warheit *et al*., 2004; Shvedova *et al*., 2005). Moreover, non-functionalized carbon nanomaterials were found to be more toxic than functionalized CNTs to *Daphnia magna* (Wang *et al*., 2016), bacteria (Fortner *et al*., 2005) fungi (Gorczyca *et al*., 2009), and fish (Wang *et al*., 2015; Zhu *et al*., 2007). In technical terms, non-functionalized or uncoated NMs are dispersed in their corresponding media rather than being dissolved (Handy *et al*., 2008a) and usually need an organic dispersant or surfactant to prevent excessive agglomeration (Smith *et al*., 2007). The difficulties of understanding the toxicity of uncoated CNTs is arguably primarily due to the inconsistency of the findings (Buzea *et al*., 2007; Gonzalez *et al*., 2008) and because the observed toxic effects have also

been attributed to the dispersants used (Oberdörster *et al*., 2004; Handy *et al*., 2008b), and residual metal impurities from the synthesis process (Oberdörster *et al*., 2005).

Several studies of CNTs agree that the shorter CNTs are more toxic to aquatic organisms than longer ones (Jia *et al*., 2005; Kang *et al*., 2009, 2008; Lawrence *et al*., 2016). Likewise, aggregate SWCNTs were found to be less toxic to bacteria than individually dispersed SWCNTs (Liu *et al*., 2009). The greater toxic effect is likely to be linked to the large specific area of smaller particles and their subsequent possible greater interaction with the membrane of an organism (Kang *et al*., 2007; Yang *et al*., 2010, Jackson, 2013).

Extensive research has been conducted to investigate CNT toxicity with a wide range of aquatic organisms, including *Daphnia* (Roberts *et al*., 2007; Kennedy *et al*., 2008; ; Kennedy *et al*., 2009; Petersen *et al*., 2009; Kim *et al*., 2010; Petersen *et al*., 2011), fish (Cheng *et al*., 2007; Smith *et al*., 2007; Fraser *et al*., 2011), algae (Wei *et al*., 2010), protozoa (Ghafari *et al*., 2008), amphibian larvae (Mouche *et al*., 2008), estuarine copepods (Templeton *et al*., 2006), and bacteria (Kang *et al*., 2007, 2008, 2009). Moreover, lipid-coated SWCNTs were found to be toxic to exposed *Daphnia*, which resulted from the deposition and clumping of CNT in the organism's intestines (Kim *et al*., 2010). In another study, several methods used to suspend the CNTs showed that higher toxicity in *Ceriodaphnia dubia* was correlated to high levels of CNT aggregation, hence suggesting that the toxicity of CNTs was related to a significant degree to the clumping of CNTs within the gut (Kennedy *et al*., 2009). Long *et al*. (2012) completed research centred on the toxicity of MWCNTs to a

freshwater green alga and showed significant algal growth inhibition. These authors found that the observed toxicity was due to increased oxidative stress, physical interaction of the CNTs with algae, and shading effects.

For example, two freshwater algae *Raphidocelis subcapitata* and *Chlorella vulgaris* showed growth inhibition following SWCNT exposure; however, no instances of death for fish *Oryzias latipes* and the crustacean *D. magna* were identified (Sohn *et al*., 2015). Hence, it is obvious that CNT exposure could induce different toxic effects as a result of the different physiology of groups of organisms, and this is notably dictated by the type of aquatic organism. For example, invertebrates and fish can ingest and accumulate CNTs in their tissues and also excrete them.

2.6 Carbon nanotube uptake, behaviour, bioavailability and bioaccumulation

Bioaccumulation occurs when an organism is taking up the chemical substance significantly more rapidly than the excretion loss of this particular substance and it requires a degree of assimilation into tissues. Low CNT bioaccumulation has been observed in various organisms, including invertebrates (Bjorkland *et al*., 2017). Moreover, recent evidence of bioaccumulation of ENMs in molluscs was reported (Rocha *et al*., 2015) and MWCNTs were also found to have accumulated in protozoa (Mortimer *et al*., 2016). Other studies observed that nanomaterials can be accumulated by some aquatic organisms much more than others (Chen *et al*., 2014). For example,

after 48 hours of exposure, C_{60} aggregates were observed in daphnia with a relatively higher accumulation than in zebrafish (Chen *et al*., 2014).

Since 2008, there has been a significant increase in studies exploring the ecotoxicity of CNTs, utilising organisms that inhabit terrestrial, sedimentary, or aquatic habitats. Broadly, although microscopy detected CNTs in the intestine of some species (Roberts *et al*., 2007; Kennedy *et al*., 2008; Mouchet *et al*., 2008; Petersen *et al*., 2010), absorption of CNTs appears to be negligible across epithelial membranes in all the examined organisms. These studies suggest that the toxic effects observed following exposure to CNTs do not seem to be perceivable in terms of absorption of CNTs through epithelial membranes in a large volume. However, not many studies have concentrated on the impact of CNTs' potential transfer or their intake through the food chain, by undertaking inclusive studies with a number of different organisms at a variety of trophic levels. For example, it was found in a small number of studies that negative impacts were identified in relation to bacteria ingestion by CNT-exposed protozoa (Zhu *et al*., 2006; Ghafari *et al*., 2008; Parks *et al*., 2013; Mortimer *et al*., 2016; Tao *et al*., 2016).

Furthermore, other studies observed that CNT sorption to sediment and soil particles was able to hinder absorption and lead to lack of uptake in organisms. For example, a significant MWCNT mass was found in the gut of *D. magna* (Petersen *et al*., 2009); however, in a separate study, the analysis of the results provided no proof that MWCNTs were absorbed by *D. magna* (Edgington *et al*., 2010). Moreover, there was no appreciable absorption (only a very small fraction $\sim 10^{-8}$ % of the total dose) into

the tissues of fruit flies (*Drosophila melanogaster*) from the gastrointestinal (GI) tract when ingesting food laced with SWCNT (10 mg/kg) (Leeuw *et al*., 2007). Generally, it seems that soil- and sediment-dwelling organisms, such as *Lumbriculus variegatus*, can readily eliminate ingested CNTs (Ferguson *et al*., 2008; Petersen *et al*., 2008; Petersen *et al*., 2010; Galloway *et al*., 2010). However, it has been observed that CNTs can also be eliminated by *Ceriodaphniadubia dubia* and *D. magna*, but only in the presence of food (Kennedy *et al*., 2008; Petersen *et al*., 2009; Petersen *et al*., 2011). As a result, one of the most important factors to consider after an exposure is the capacity of a given organism for elimination of the substance. There is little likelihood of biomagnification occurring via the food chain, as experimental work has found that organisms can in general quickly and efficiently eliminate CNTs throughout the course of depuration (Bjorkland *et al*., 2017).

Petersen *et al*. (2008) investigated sediments spiked with CNTs in order to investigate the kinetics of uptake and depuration. They synthesised radio-labelled SWCNTs and MWCNTs using the chemical vapour deposition procedure and introduced them into sediment samples in order to assess their uptake by a sediment-burrowing oligochaete *L. variegatus*, and reported low bioavailability. Their results showed that, compared to the control sediments, the overall number of *L. variegatus* was not seen to decrease following a period of 28 days of exposure to sediments spiked with SWCNTs (0.03 or 0.003 $mg.g^{-1}$) and MWCNTs (0.37 or 0.037 $mg.g^{-1}$). Depuration behaviours observed by examining cellular tissues suggested that SWCNTs and MWCNTs were not absorbed into cellular tissues, but they were rather seen to be associated with residual

sediment matter within the gut. The findings implied that organism tissues do not easily absorb purified carbon nanotubes. With regard to the uptake and eradication of CNTs, it appeared that, in the case of the organisms under examination, nanotubes were found to be from sediment that had yet to be expelled from the guts of the organism (Petersen *et al*. 2008). Two days following depuration, the worms were observed to have removed the CNTs, when positioned in clean sediment; however, in water, the elimination proceeded at a slower rate (Petersen *et al*., 2008).

2.7 Ecotoxicological Effects of CNTs in sediments

A number of different studies carried out in relation to the concentration of CNT have reported very low ecotoxicological results when examining sediment and soil (Galloway *et al*., 2010). Such works have emphasized that the presence of sediment reduces the observed effect resulting from the sorption/attachment interactions of CNT with particles of sediment (Petersen *et al*., 2008). For example, lugworm (*Arenicola marina*) feeding rates and burrowing behaviour were not affected by exposure to sediment spiked with SWCNT (0.003 g/kg), nor was there any evidence of a negative effect on DNA, as assessed using the comet assay (Galloway *et al*., 2010). Moreover, when the concentration of CNTs in food was in excess of 37 mg double-walled CNT (DWCNT)/kg, the earthworm reproduction rate was seen to fall (*Eisenia veneta*). However, concentrations of up to 495 mg DWCNT/kg food did not impact their hatchability and survival (Scott-Fordsmand *et al*., 2008). Similarly, sediment concentrations as high as 3 g/kg of MWCNTs and SWCNTs across the two different

soils did not impact the lipid dry mass or content of earth worms (Petersen *et al*., 2008). These studies indicate that the observed toxic effects of CNTs may be influenced by various effects exhibited upon organisms' surfaces. Moreover, there were no recognized impacts in regard to the biodiversity of the benthic macroinvertebrate community following the addition of up to 2 g/kg concentration of MWCNTs to sediment, which actually led to significant increase in benthic macroinvertebrate communities (Velzeboer *et al*., 2011). In contrast, nitrogen and carbon microbial biomass were found to decrease MWCNTs in soil, with a decrease also witnessed in the case of enzymatic activity at 5g/kg; however, statistically significant effects were not established in the case of a CNT concentration of 0.5g/kg in soil (Chung et al., 2011).

Galloway *et al*. (2010) compared the potential of SWCNTs and nanoTiO_2 in marine sediments to present sub-lethal toxicity to the infaunal polychaetae worm *Arenicola marina* (lugworm). They detected SWCNTs through the application of different methods, namely TEM and coherent anti-Stokes Raman scattering microscopy approaches. These two methods suggest that SWCNTs are not able to traverse the epithelial membrane in large volumes, leading to starvation and clearance of sediment-associated SWCNTs from the gut (Galloway *et al*. 2010). Moreover, as expected, by using TEM, it was confirmed that both SWCNTs and nanoTiO_2 formed aggregates (Galloway *et al*. 2010). Following a 24-hour period of starvation, it was observed that the NMs could pass through the gut, at which point they were excreted, or remain within the sediment, as no SWCNTs were identified as present in the lumen of the gut

(Galloway *et al*. 2010). Raman scattering microscopy was used to detect TiO_2 within the gut lumen and adhered to the external epithelium of the worms (Galloway *et al*. 2010). Although there was no visible observation of particle uptake into tissues, Raman scattering microscopy located TiO_2 aggregates (>200 nm) within the gut lumen and were also seen to bind to the external epithelium of the worms. After conducting the tests for toxicity, which importantly spanned a period of 10 days, no key impacts were identified on any outcomes in the case of SCWNTs at a concentration of 0.03 g/kg (Galloway *et al*. 2010). Similarly, no uptake of SWCNTs into tissues was seen (Galloway *et al*. 2010). However, high rates of both DNA damage ($P = 0.008$) and also cellular damage ($P = 0.04$) to coelomocytes were observed, in line with increasing concentrations of nanoTiO_2, with preliminary lowest observed effect concentration (LOEC) of 1 g/kg (Galloway *et al*. 2010). The findings suggested that while sediment-associated SWCNTs were not shown to cause toxicity to the lugworm at concentrations of up to 0.03 g/kg, an interaction was observed between the lugworm and sediment-associated SWCNTs (Galloway *et al*. 2010).

In an extensive study, Parks *et al*. (2013) assessed the importance of the exposure route in affecting the bioavailability and bioaccumulation of SWCTs in the mysid *Americamysis bahia*, the marine amphipod *Ampelisca abdita*, and the estuarine amphipod *Leptocheirus plumulosus*, by spiking either food matrices or sediments. They found that, although SWCNTs were bioaccessible to those marine organisms, they did not seem to cause toxicity nor was there any instance of death identified through either route (with the inclusion of food media or via sediment), at

concentrations as high as 100 µg/g. In addition, they also suggested that the accumulation of SWCNTs in *Leptocheirus plumulosus* was more noticeable in the uptake route of sediment ingestion than the uptake route via food exposure (Parks *et al*., 2013).

SWCNTs were not detected in the depurated organisms, but were quantified in non-depurated *A. abdita* that had been fed algae *Cyclotella meneghiniana* previously exposed to SWCNT (Parks *et al*., 2013). Following a 28 day exposure to SWCNTs-spiked algae (100 µg/g), as well as SWCNT-spiked sediment (100 µg/g), SWCNTs were identified in both non-depurated and depurated *L. plumulosus* amphipods at 5.38 µg/g and 0.50 µg/g, respectively (Parks *et al*., 2013). The findings suggested that although SWCNTs were not appearing to accumulate or cause toxicity, they did interact with benthic organisms.

To summaeise, toxic effects of CNT in aquatic organisms can be varied. CNTs are not toxic for aquatic organisms at environmentally relevant concentrations. Moreover, CNT toxicity effect was only observed at high concentrations. Furthermore, CNT ecotoxicity depends on the exposition time, type of organisms, and CNT preparation methods. CNT can modify the other contaminants' toxicity.

2.8 The Influence of Contaminants on the CNTs Toxicity

CNT research has recently been extended to determine their interaction with pesticides, metals, pharmaceuticals and surfactants, which could potentially lead to

changes in the toxicity of CNTs in aquatic ecosystems. Several studies have attempted to clarify the potential impact of CNTs on the availability of other contaminants in estuarine invertebrates *Amphiascus tenuiremis* and *Streblospio benedicti* (Ferguson *et al*., 2008), earthworms *Eisenia fetida* (Petersen *et al*., 2009) and on humans, such as through inhalation (Helland *et al*., 2007; Poland *et al*., 2008). Although the effect of contaminants and NMs in the environment are difficult to predict, several studies have found significant interactions (decreasing or increasing the toxicity) once contaminants are exposed to or associated with CNTs. This antagonistic response suggests that the contaminants may be bound by the CNTs, causing them to be sequestered by the CNTs and, as a result, possibly decreasing their overall bioavailability, and thus, their toxicity to biota. For instance, a number of studies have highlighted a decrease in toxicity following the incorporation of (i) MWCNTs in addition to Cu in rotifer (*Brachionus koreanus*) exposures (Lee *et al*., 2016) or (ii) SWCNTs in combination with PCB in the case of benthic invertebrates such as *L. plumulosus* (Parks *et al*., 2014). On the other hand, different CNTs were found to increase cadmium and Cu ecotoxicity in *D. magna* (Wang *et al*., 2016). There are only a few *in vivo* studies that have examined the behaviour of CNTs in marine systems and their bioavailability and toxicity to marine organisms (Templeton *et al*., 2006; Pacurari *et al*., 2008; Woods *et al*., 2009; Galloway *et al*., 2010; Mwangi *et al*., 2012; Al-Shaeri *et al*., 2013; Girardello *et al*., 2015; Lukhele *et al*., 2015). Al-Shaeri *et al*. (2013) concluded that relatively low concentrations of SWCNTs , at level of 5mgL^{-1} -50mgL^{-1}, have a potentiating effect on the toxicity of otherwise benign concentrations

of secondary metal contaminants and therefore pose an indirect risk to aquatic organisms. In their study, Al-Shaeri *et al*. (2013) explored SWCNTs ecotoxicology and their predicted contact with metals that had been dissolved in seawater. The main focus was the CNTs bioavailability, as well as the influence of in vivo exposure to CNTs by marine filter-feeding bivalves, *Mytilus edulis.* The mussels were spiked with SWCNTs alone, $CdCl_2$ alone, zinc alone, $CdCl_2$ + zinc, SWCNTs + $CdCl_2$, SWCNTs + zinc, or SWCNTs + $CdCl_2$ + zinc at different concentrations (5mgL^{-1}, 10mgL^{-1}, 50mgL^{-1}, 100mgL^{-1}, and 500mgL^{-1}) for 72 h (Al-Shaeri *et al*., 2013). No significant negative effect on DNA was identified, nor increased oxidative stress, based on measurements carried out on mussels exposed *in vivo* to SWCNTs alone, at up to 50 mgL^{-1}, whereas DNA and oxidative stress increased significantly after exposure to concentrations of 100mgL^{-1} and 500mgL^{-1}. Moreover, *M. edulis* exposed to either $CdCl_2$ or zinc and both metals combined did not incur DNA damage and oxidative stress in excess of the control group (Al-Shaeri *et al*., 2013). However, when examining exposure to SWCNTs (5–500mgL^{-1}) alongside metals ($CdCl_2$ or zinc), a significant rise in the degree of damage to DNA was observed, as well as oxidative stress, above the effects of exposure to the SWCNTs or metals alone (Al-Shaeri *et al*., 2013).

Taken together, the above-mentioned studies indicate that there was no discernable pattern in the interactions between CNTs and metals, as these significantly rely on each contaminant's physiochemical aspects.

2.9 Experimental Organisms

2.9.1 Bivalves as Bioindicators for Ecotoxicological Monitoring

There are several key factors that need to be considered when selecting a test organism to assess sediment toxicity, such as its availability, ecological relevance, sensitivity to contaminants, method of feeding, whether the organism is sessile or transient and its exposure history. Bivalves have been applied to assess the effects observed in aquatic settings in relation to both anthropogenic and natural disturbances. This is particularly due to such environments' somewhat fast-paced reaction to stresses, of both anthropogenic and natural causes, together with their respective aspects of life-history. In addition, this interest is due to the various features of the benthic community, including the richness of the species, as highlighted in various studies (Griscom *et al*., 2002; Cheggour *et al*., 2005; Machreki-Ajmi and Hamza-Chaffai, 2006; Calabretta and Oviatt, 2008; Velez *et al*., 2016). Various studies have been carried out in relation to pollution across the environment, with much emphasis placed on bivalves as biomarkers, as listed in the following table (Table 2.3). Acting as sentinel organisms, their deposit feeding and sedentary suspension as well as their capabilities of filtering of large amounts of water, means that bivalves are able to garner significant volumes of heavy metals and, accordingly, adopt the role of metal contamination bioindicators in the marine environment (Cheggour *et al*., 2001; Griscom *et al*., 2002), and can therefore help to detect marine pollution (Cheggour *et al*., 2001; Griscom and Fisher 2004).

Using biological indicators to monitor trace toxic substances in aquatic media is well understood. Bivalves are often considered as a good bioindicator species because of their sessile approach to life, long life-spans, ease of sampling, wide geographical distribution, tolerance of differing salinities, and resistance to high accumulations of a variety of chemicals (Tanabe *et al*., 1987; Hamza-Chaffai, 2014; Valavanidis *et al*., 2006). Consequently, when assessing environmental pollution, a widely used bivalve is the cockle *C. edule* (Velez *et al*., 2016). Cockles have been used as biological "sentinels" in environmental monitoring studies because of their tendency to uptake metals from the surrounding media and concentrate them (Mat *et al*., 1994a, b; Noorddin, 1995; Chan *et al*., 2002).

Table 2.3: List of Selective Pollution Studies Focused on Bivalves

Bivalve's Latin Name	Contaminantes	Bimarker/Endpoint	Studies
Anadara granosa and *Perna viridis*	Cd ≈ 2.20 Cr ≈ 8.35 Cu ≈ 5.75 Pb ≈ 1.75 Se ≈ 2.1 Zn ≈ 90.5	Neutron activation analysis (NAA) and atomic absorption spectrophotometry (AAS)	The assessment of environmental poullution of the coastal areas o the Malaysian Peninsula (Yusof *et al*., 2004)
Ruditapes decussatus	Cd 40 μg l^{-1}	Micro liquid chromatography tandem mass spectrometry (LC–MS/MS).	Cd effects in the gill and digestive gland (Chora *et al*., 2009)
Ruditapes decussatus	Cd, ≈ 0.52 μg.g^{-1} Cu, ≈ 1.5 μg.g^{-1} Zn ≈ 45 μg.g^{-1}	Atomic absorption spectrophotometry, differential pulse polarographic assay, and thiobarbituric acid-reactive substances	To assess the health status of *R. decussatus* in a contaminated marine ecosystem (Hamza-Chaffai et al., 2003)

Mytella guyanensis	Cd ≈ 0.91 μg.g^{-1} ww Cr ≈ 0.42 μg.g^{-1} ww Pb ≈0.41 μg.g^{-1} ww Cu ≈ 7.29 μg.g^{-1} ww	Thiobarbituric acid-reactive substance (TBARS) and superoxide dismutase	Evaluate the trace metal contents and the effect of pollution on mussel (Torres *et al*., 2002)
Mytilus galloprovincialis	fluoranthene (FLU) 3 μg/L 125 μg/g dw. 60 μg/L dose of 2500 μg/g dw	Oxidative stress, phenoloxidase, and physiological rates.	Identify the effect of one of these variables, the food availability, and consequently, the mussel nutritive status (e.g. González-Fernández *et al*., 2015)
Cerastoderma glaucum	Cd ≈ 0.82	oxidative stress	Assessment of sediment/water contamination (Machreki-Ajmi and Hamza-Chaffai, 2008)
Cerastoderma edule	Cd ≈ 0.6 Cu ≈ 25.5 Pb ≈ 13.5 Zn ≈ 87.0	Atomic Absorption Spectrophotometry	To ssess the *Cerastoderma edule* would be useful as a biomonitor in some restricted environments (Cheggour *et al*., 2001)
Cerastoderma glaucum	4-nonylphenol (NP) 0.0125, 0.025, 0.05 and 0.1 mg/L	Intracellular superoxide anion assay, Lysozyme-like activity assay and Acid phosphatase activity assay.	Immunotoxicity of the xenoestrogen 4-nonylphenol to the cockle (Matozzo et al 2008)
Anadara granosa	carcinogenic polycyclic aromatic hydrocarbons (t-PAHs) ≈ 20 ng/g ww	The risk assessment of probable human carcinogens in the Group B2 PAHs was calculated and assessed in accordance with the standards of the United States Environmental Protection Agency (US EPA)	Risk assessment for the daily intake of polycyclic aromatic hydrocarbons from the ingestion of cockle (Anadara granosa) and exposure to contaminated water and sediments along the west coast of Peninsular Malaysia (Mirsadeghi et al., 2011)

In recent years, the body of literature studying the toxicity of CNTs has grown considerably, using cell culture and animal models, mainly to address the risks to human occupational health (Al-Shaeri *et al*., 2013; Khalid *et al*., 2016). According to Al-Shaeri *et al*. (2013), SWCNTs have a potentiating effect on the action of the metals

on the mussel *M. edulis* and are harmful to marine organisms in the laboratory and may therefore be harmful to the marine environment. Although there is a lack of clarity regarding uptake of NMs they may be taken via the gill epithelium, across the epithelium of the digestive tract, or the hepatopancreas, where nutrient storage and absorption occur in invertebrates (Sahu and Casciano, 2009).

2.9.2 Cockles as Bioindicators for Ecotoxicological Monitoring

The edible cockle *C. edule* (Linnaeus, 1758), is a widely distributed and common bivalve species on mudflats and sandy/fine gravelly bays of tidal coasts of European countries, where they frequently play an important commercial role and an important food source for other species (Tebble, 1966; Sanchez-Salazar *et al*., 1987; Callaway *et al*., 2014; Magalhães *et al*., 2018). Its distribution area extends from the Barents Sea in the North to the West Coast of Africa in the south (Callaway *et al*., 2014), and its wide range of tolerance to salinity means it is a common estuarine species (Tyler-Walters, 2007) (Table 2.4). *C. edulis* are abundant in Scotland, and they are especially prevalent in Cramond, in the Firth of Forth, where the specimens for the current work were collected.

Table 2.4: Descriptive Information about Cockle (*C.edule*), adapted from Tyler-Walters (2007).

Description and identifying features:	
• Thick shell, equivalve, solid, globular and broadly oval in outline; ≤ 5 cm long, with front of midline beaks (anterior). • 22-28 radiating ribs shell, bear short flat spines and crossed by conspicuous concentric ridges. • External ligament. • Yellowish to brownish outer surface or off-white. • Prominent growth lines. • Dull white inner surface, with a light purple or brownish stain on or about the posterior adductor muscle scar. • Pallial line lacks a sinus. • Each of the two valve bear cardinal teeth. • The left valve bears a single anterior and posterior lateral while the right bear two anterior and two posterior lateral teeth. • Shallow grooves on the inner surface run from the notched margin, fading before reaching the pallial line.	
Global distribution:	
• Western Barents Sea and northern Norway to the Iberian Peninsula, and south along the coast of west Africa to Senegal. • Estuaries and sandy bays around the coasts of Britain and Ireland.	
Habitat:	
Inhabits the sediments surface, digging to a depth of ≤ 5 cm. Found on clean sand, muddy sand, mud or muddy gravel from the middle to lower intertidal, sometimes subtidally. Live at 15 -35 psu salinities but can tolerate salinities to 10 psu. Often abundant in estuaries and sheltered bays, and recorded population densities of 10,000 per m^2.	
Taxonomy:	
Phylum	Mollusca - Snails, slugs, mussels, cockles, clams & squid
Class	Bivalvia - Clams, cockles, mussels, oysters, and scallops
Order	Cardiida
Family	Cardiidae
Genus	Cerastoderma

Authority	Linnaeus (1758)
Recent Synonyms	Cardium edule (Linnaeus, 1758)
Biology:	
Typical	Abundance in high density
Male size range	ca. 3 -38mm
Male size at maturity	15-20mm
Female size range	15-20mm
Growth form	Bivalved
Growth rate	Variable.
Body flexibility	None (less than 10 degrees)
Characteristic feeding method	Active suspension feeder
Typically feeds on	Phytoplankton, zooplankton and organic particulate matter.
Environmental position	Infaunal
Supports	Host / The parasitic copepod Paranthessius rostatus, and the larval stages of various species of digenetic trematode.
Is the species harmful?	No Edible
Life history:	
Reproductive type	Gonochoristic (dioecious)
Reproductive frequency	Annual protracted
Fecundity (No. of eggs)	>1,000,000
Generation time	1-2 years
Age at maturity	18 months
Season	May - June
Life span	5-10 years
Fertilization	External
Spawning time	Between March - August in the UK followed by peak spatfall between May and September.

The genus *Cerastoderma* is comprised of four species *C. elegantulum, C. lamarcki*, *C. glaucum, and C. edule*. The cockle (*C. edule*) is the most common one. These organisms actively move in their environment, modifying the chemical and physical properties of their habitat by reworking the sediment (Hedman *et al*., 2011). The influence of such species on sediment remains a subject of debate. Through their valve movement, they are increasing the fluxes of suspended material and the sediment erosion as they modify the top layer of the sediment. They are filter feeders, through which they remove the fine sediment from the water column (Rakotomalala *et al*., 2015).

The stabilizing (Andersen *et al.,* 2010) or destabilizing (Ciutat *et al.,* 2006, 2007) effect of cockles on sediment has been debated. The cockle burrows using its muscular foot, which allows it to burrow deep into the sediment to seek refuge from abiotic disturbance (waves) and predation (Figures 2.8 and 2.9). Importantly, according to Ramon (2003), in some places, cockles make up as much as 60% of the benthos biomass and play a key role in the overall operation of the intertidal ecosystem (Beukema and Dekker, 2006).

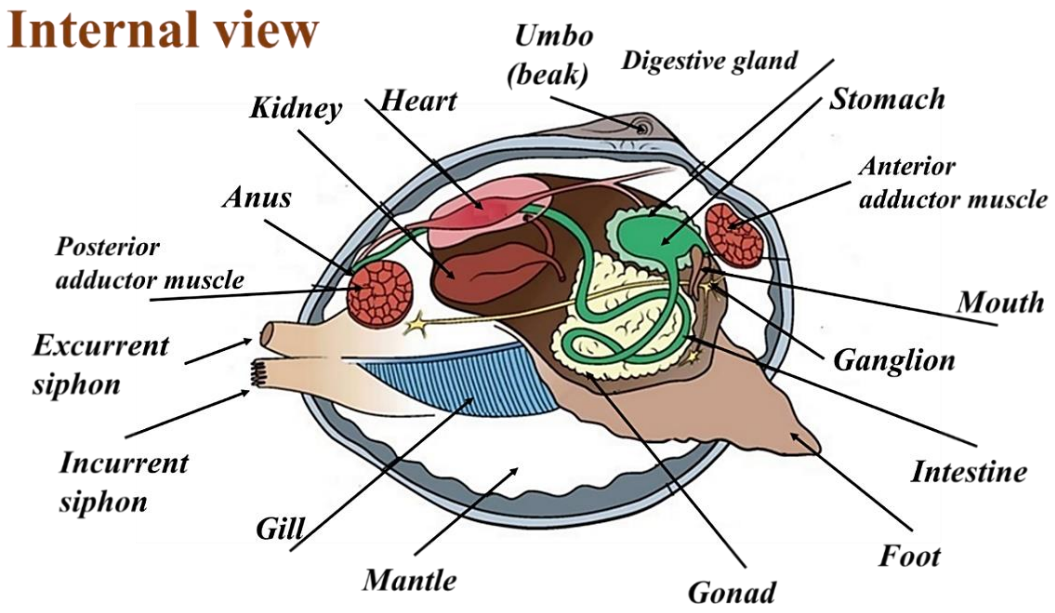


Figure 2.8: The morphology of the cockle (*C. edule*). Adapted from Mollusca Anatomy (2018).

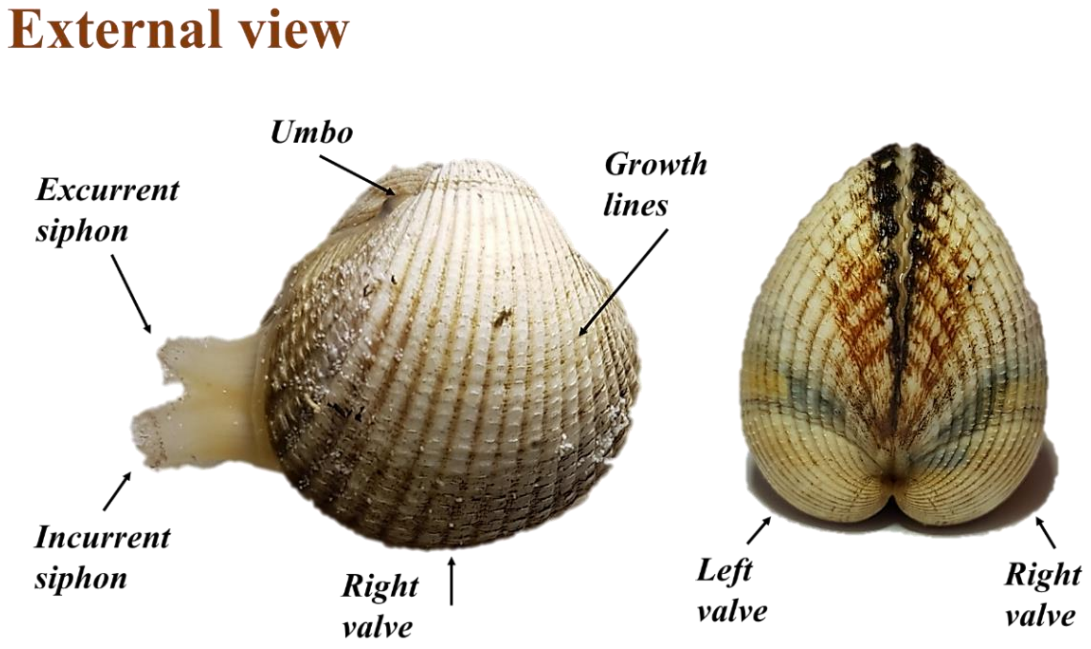


Figure 2.9: The external morphology of cockle (*C. edule*).

2.10 Biomarkers and Approaches

2.10.1 Cell viability

One of the most fundamental biomarkers used in studying cytotoxicity is cell viability, which is established through measuring the numbers of living or dead cells, usually through the application of particular dyes, as explained in the work of Davey & Kell (1996). Cell viability approaches have been considered as an indicator of general biological responses (Youn *et al.*, 2012). There are several reasons why cell viability is an essential tool in studies of cytotoxicity. Firstly, cell viability levels are good indicators of cell health. Secondly, the measurement of cell viability is evidence of whether the treatment has killed the cells or kept them alive. Lastly, it might be complicated to perform other endpoint studies on dead cells, such as oxidative stress and comet assays. There are different approved assays for cell viability. For example Trypan blue that uses a light microscope and utilises the exclusion of particular dyes by live cell membranes, propidium iodide (Pi) using flow cytometry, tetrazolium bromide (MTT) dyes that measure the mitochondrial dehydrogenase activity in cells, while crystal violet (CV) and neutral red (NR) stains are primarily used for assessing the integrity of lysosomes and cell membranes (Jones and Senft, 1985; Pulskamp *et al*., 2007).

Binelli *et al*. (2009), measured the overall TCS (Cytotoxicity of Triclosan) in relation to haemocytes of the freshwater zebra mussel (*Dreissena polymorpha*) as a preliminary marker of any later-identified genotoxicity. Nonetheless, TCS are

recognised as being rather time-inefficient and labour in tensive. In recent decades, a number of different approaches have been devised to evaluate and measure biological cells, including FC (Flow Cytometry) and Trypan Blue.

2.10.1.1 Trypan blue

Trypan blue is viewed as being a simple and standard approach, which can be applied in order to evaluate the overall viability of cells through taking cells and staining them; these are then examined under a microscope within a three-minute period; the dead cells will take up the stain but the viable cells do not (Absolom, 1986). The cell viability of the haemocytes of mussel (*M. edulis*), after immersion in either Hanks Balanced Salt Solution (HBSS) or Leibovitzmedium (L-15), has been successfully measured using Trypan blue (Hartl *et al*., 2009). In the current study, the Trypan blue technique was employed to assess cell viability in cockle haemocytes, as this technique is simple to use, involves less cost and provides reliable results, in addition to the small number of cells required (Absolom, 1986).

2.10.2 Comet Assay or Single Cell Gel Electrophoresis (SCGE)

When examining a cell's molecular make-up, one of the most fundamental environmental stress markers across both aquatic and terrestrial organisms is that of DNA. Affecting the DNA's integrity could subsequently result in mutations, which might lead on to disease, including cancer in vertebrates, as well as a number of additional irreversible toxic impacts identified in various works (Sina *et al*., 1983;

Kadhim and Parry, 1984; De Flora *et al*., 1991; Kurelec, 1993; Bailey *et al*., 1996; Steinert, 1999) as the 'genotoxic disease syndrome' amongst invertebrates (Figure 2.10).

A number of different methods have been identified as suitable when evaluating damage to DNA, which include CA (the chromosomal aberration assay), SCE (the sister-chromatid exchange assay) and MNT (the micronucleus test) (Kim and Hyun 2006). In particular, metaphase methods, for example CA and SCE, have not been found to be useful for detecting damage *in vivo*, because only a few cells simultaneously occur in metaphase and there are also often karyotype limitations, particularly in triploid fish species (Hooftman and de Raat, 1982). For example, for a number of fish species, including Cyprinids and Salmonids, the metaphase approach is unsuitable, because a significant number of small and irregular chromosomes are present in a Fish karyotype, which may be triploid, for example (Al-Sabti and Metcalfe, 1995). Thus, not all assays are suitable for application across an extensive number of cells, with some manipulated specifically for use with mammalian cells.

Comet assay is regarded as being a valuable and in-depth approach to DNA damage evaluation; the term 'comet' is used in this regard to explain the individual cell DNA migration pattern observed through this approach, with a long 'tail' (Tice *et al*., 2000). Comet assay has been widely used to detect environmental mutagens affects, to assess irradiation damage (UV; solar radiation and X-rays) and compound-induced genotoxicity. Failure to repair DNA lesions can initiate a cascade of biological reactions with potential consequences at organ, cellular, whole animal and eventually

at the population and community levels. DNA damage may cause by a variety of DNA damage agents or responses (Figure 2.10) (Lee and Steinert, 2003). The comet assay has become one of the most popular techniques used to detect DNA damage, expressed as DNA strand breaks at the level of individual cells (Klobučar *et al*., 2003; Lee *et al*., 2003). The comet assay has been applied in various applications in order to detect the effects of environmental mutagens and to assess irradiation damage and compound-induced genotoxicity. The assay has become an effective tool for bio-monitoring, because of its increased refinement and use for environmental purposes (Hartl *et al*., 2010). It is easy to use, quick, flexible and sensitive, enabling detection of a small amount of DNA damage, and thus requiring a very small number of cells (50-100 per sample) for the experiments, and providing reliable findings.

This approach was first presented in the study carried out by Östling and Johanson (1984), with the aim of identifying double-stranded breaks in DNA in individual cells (Fairbairn *et al*., 1995; Rojas *et al*., 1999; Tice *et al*., 2000). The cells are embedded in agarose then placed onto a microscope slide. The cells are lysed by detergents and salt treatment and the liberated DNA electrophoresed under neutral conditions (pH 7), which helps to ensure double-stranded DNA breaks can be identified in isolation (Rojas *et al*., 1999; Tice *et al*., 2000).

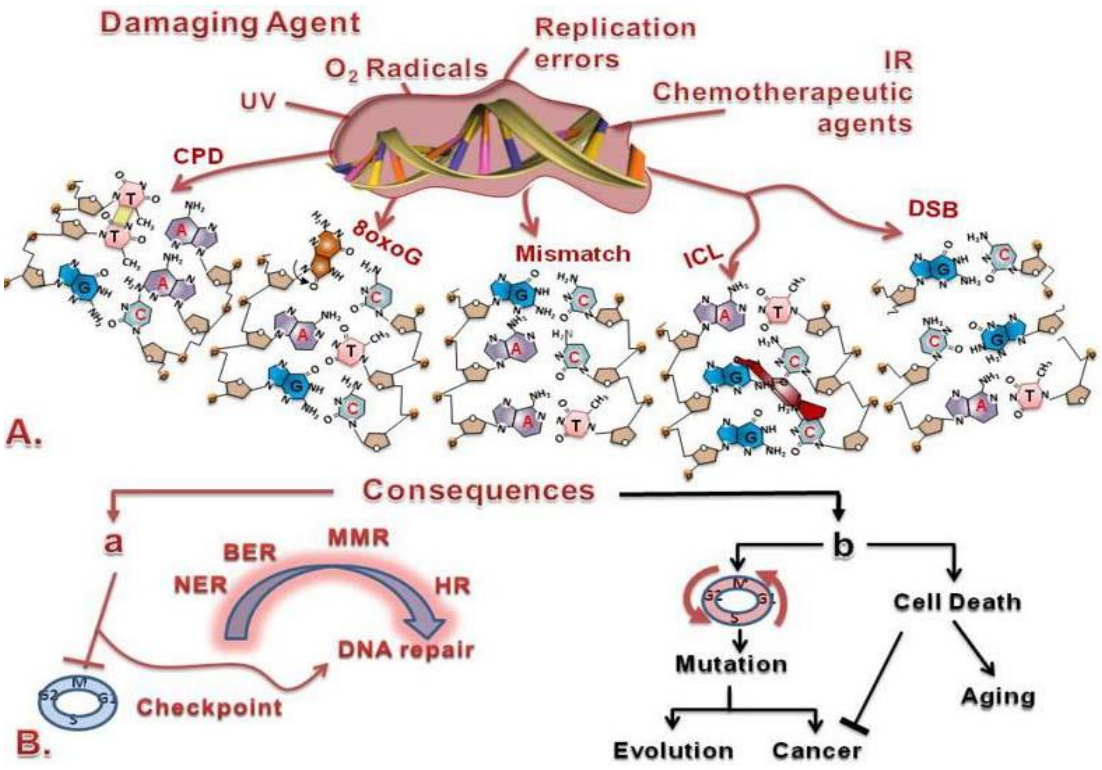


Figure 2.10: DNA damaging agent (A) and long-term consequences of DNA lesions (B) (Moraes *et al.*, 2012).

Singh *et al*. (1988) expanded this approach, via the adoption of Alkali-labile sites (ALS) (pH >13), focused on identifying and examining single-stranded DNA breaks (SSBs); this technique has been referred to by many researchers (Singh *et al*., 1988; Fairbairn *et al*., 1995; Collins *et al*., 1997; Rojas *et al*., 1999; Tice *et al*., 2000) as SCGE (Single Cell Gel Electrophoresis). In more recent years, a number of changes and enhancements have been applied to Comet assay versions, making this a useful approach for testing the cells of a number of different species, such as bivalves, and a number of other invertebrates (Woods *et al*., 1999; Klaude *et al*., 1996; Coughlan *et al*., 2002; Rank and Jensen 2003; Hartl *et al*., 2004; Hartl *et al*., 2010). Despite Comet

assay beneficiary, however, if there are larger number of samples (more than 50 samples), it becomes complicated and may take longer to obtain findings (Woods *et al*., 1999; Tice *et al*., 2000; Singh and Hartl 2012).

The increased adoption of the comet assay and the subsequent changes made to the technique to pursue environmental objectives have established the comet assay as a fundamental and valuable instrument in the bio-monitoring arena (McCarthy and Shugart, 1990, Belpaeme *et al*., 1996; Coughlan *et al*., 2002; Hartl *et al*., 2010). This method of assessing damage to DNA has previously been directed towards evaluating the genotoxicity effect water-borne pollutants in the case of both freshwater and marine bivalves (Nacci *et al*., 1996; Pavlica *et al*., 2001; Coughlan *et al*., 2002; Hartl *et al*., 2004). The comet assay approach has been further developed by Wilson *et al*. (1998) in a study by with the aim of examining single-cell suspensions derived from mussels' digestive glands and gills of Rank (1999), who discussed the application of comet assay in identifying damage to DNA in gill and haemolymph cells. In addition, subsequent developments have been implemented to the assay, notably in the work of Hartl *et al*. (2010) and Singh & Hartl (2012). Importantly, the link between the integrity of DNA and its vulnerability in terms of oxidative stress in mussels (*Mytilus galloprovincialis*) has been examined in the research of Frenzilli *et al*. (2001), who examined the notably greater degree of oxidative stress and damage to DNA amongst some bivalve specifically those gathered from inner parts (the highly eutrophicated Orbetello Lagoon) compared to specimens from more external sites.

The comet assay method has been commonly acknowledged and valued in a number of different field research and laboratory settings as a result of its adoption for studying cells of various organisms, including humans, mussels and fish (for example, Singh and Hartl, 2012). Furthermore, the comet assay approach has a number of benefits, including ease of use, the potential to complete work with a quite small volume of the test substance, flexibility, simplicity, inexpensiveness, sensitivity in evaluating low levels of DNA damage, reliable findings, and the short timescales needed to carry out experiments (Woods *et al*., 1999; Collins *et al*., 1997; Tice *et al*., 2000; Hartmann *et al*., 2003; Akcha *et al*., 2004; Klobucar *et al*., 2008; Dhawan *et al*., 2009; Singh and Hartl, 2012).

The comet assay method incorporates a number of different stages, primarily involving embedding a suspension of nucleated single cells (may require one stage centred on extracting cells from complicated organ tissues) within an agarose sandwich (NGA and LMP). This involves taking a microscope slide and putting it into a lysis solution to removing the cell membranes, thus implementing the conditions for electrophoresis; this is in order to allow the DNA to become unwound. Subsequently, neutralisation is carried out to enable staining with Gelred, and DNA damage then measured under an epifluorescence microscope using live video scoring software (Woods *et al*., 1999; Rojas *et al*., 1999; Tice *et al*., 2000; Hartmann *et al*., 2003; Singh and Hartl 2012).

In summary, the comet assay approach has been the focus of further enhancement and development to become an innovative instrument for assessing damage to DNA, both *in vitro* and *in vivo*, across a wide range of different cells taken from numerous types

of organisms, such as fish (Kilemade *et al*., 2004; Hartl *et al*., 2007; Monteiro *et al*., 2011; Pereira *et al*., 2010), as well as species of invertebrates (Mitchelmore and Hyatt, 2004; Lewis and Galloway, 2008), including bivalves (Coughlan *et al*., 2002; Rank and Jensen, 2003; Hartl *et al*., 2004; Richardson, 2008; Pisanelli *et al*., 2009; Hartl. *et al*., 2010).

2.10.3 Superoxide Dismutase (SOD)

ROS (Reactive Oxygen Species) are continuously produced as a side-effect stemming from cell respiration mechanisms in organisms; this could potentially result in a number of different cell effects, with the inclusion of, for example, oxidation of DNA bases, lipid peroxidation, the degradation of protein, and enzyme inactivation (Halliwell, 1993; Zelko *et al*., 2002; Nozik-Grayck *et al*., 2005; Almeida, 2005). Various antioxidant defence enzymes have been developed by cells to safeguard them from the effects of ROS creation, including the use of protective compounds, such as Vitamin C, and metal sequestration, amongst others (Fattman *et al*., 2003).

SOD is an antioxidant defence enzyme that can provide the fundamental and necessary safeguard to biological cells against the uncontrolled reactions of oxygen-based radicals; to achieve this it acts as a catalyst to prevent mutations by superoxide (O_2) radicals, by converting them to hydrogen peroxide (H_2O_2) and oxygen (O_2) (Keller *et al*., 1991; Crapo *et al*., 1992; Fattman *et al*., 2003). At the present time, when examining living cells, three SOD enzyme isoforms are able to be established, which

are categorised according to the metals they contain, as well as their function, localisation and type of amino acid sequence (Figure 2.11).

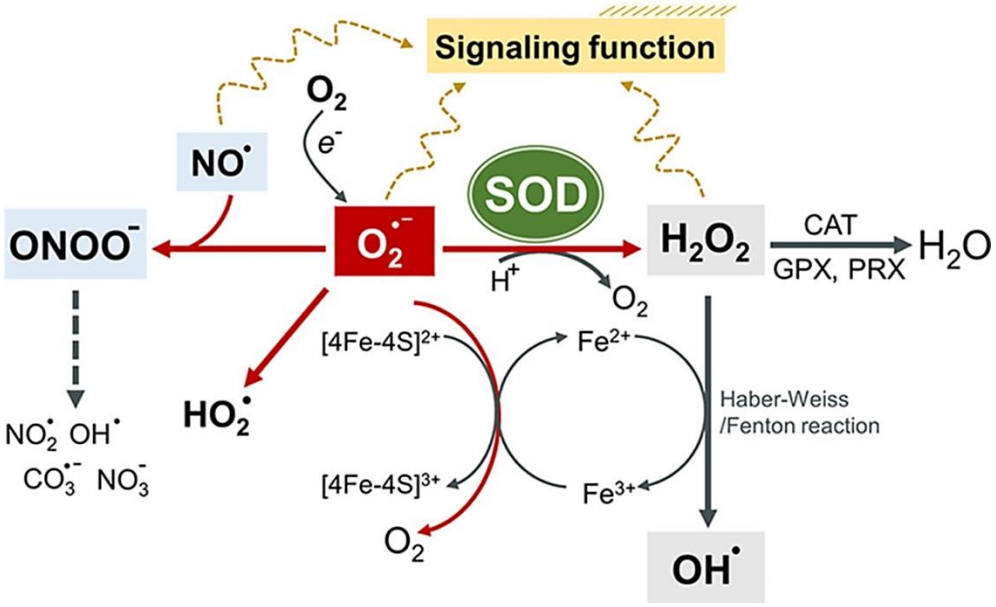


Figure 2.11: Reactions and transformations of the superoxide anion (Wang *et al.*, 2018)

In the study of McCord and Fridovich (1969), there was the establishment of a SOD-type enzyme, referred to as SOD 1 (Cu zinc superoxide dismutase) (Cu/ZnSOD). It was determined throughout the course of the work that the dismutation of the superoxide radical into hydrogen peroxide (H_2O_2) and oxygen (O_2) is catalysed by SOD1 (Carpo *et al*., 1992; Zelko *et al*., 2002; Nozik-Grayck *et al*., 2005). Cu/ZnSOD is widely recognised as a key intracellular SOD and is commonly distributed through the cytoplasm of all mammalian cells, the mitochondrial intermembrane space, and the nucleus and peroxisomes, as in the cases of human beings, mice and deer, for

example, in addition to being present within the periplasmic space of bacteria lysosomes (Keller *et al*., 1991; Fattman *et al*., 2003; Valentine *et al*., 2005).

SOD1 may be defined as a protein that is made up of two identical polypeptide chains, referred to as homodimers, comprising one zinc ion and one Cu ion across each respective 16-kDa subunit of 153 amino acids. Those ions of Cu^{2+} are confined through interaction with the histidine residue imidazolate ligands, in the case of SOD1, at the active enzyme location. Importantly, enzyme stabilisation is assisted through the Zn^{2+}. The following reaction provides an overview of the SOD1 action mode; its activities are almost independent of pH in the range of 5.0 to 9.5 and at physiological pH the reaction rates are approximately diffusion-limited (~109 M^{-1} s^{-1}) (Valentine *et al*., 2005; Liochev and Fridovich, 2007; Miyamoto *et al*., 2010;):

$$Cu^{2+}/Zn\text{-}SOD + O_2^{\bullet -} \rightarrow Cu^{2+} / Zn\text{-}SOD + O_2$$
$$Cu^{2+}/Zn\text{-}SOD + O_2^{\bullet -} + 2H^{+} \rightarrow Cu^{2+}/Zn\text{-}SOD + H2O2$$
$$2O_2^{\bullet -} + 2H^{+} \rightarrow O_2 + H2O2$$

SOD2 or manganese superoxide dismutase (MnSOD) is another type of enzyme of the SOD family. It is expressed as a tetramer containing a leader peptide in the endoplasmic reticulum, mitochondrial, secretory vesicles, Golgi elements and nuclear envelope of mammalian cells (such as human, rat and mouse) (Crapo *et al*., 1992; Zelko *et al*., 2002; Nozik-Grayck *et al*., 2005). Marklund *et al*. (1982) detected a further new SOD enzyme, which they named superoxide dismutase (EC-SOD or

SOD3). This SOD enzyme is an extracellular superoxide dismutase with a molecular weight of 135,000 kDa and minor species-specific variations (in human, rat, rabbit and mouse) (Fattman *et al*., 2003; Nozik-Grayck *et al*., 2005). EC-SOD also contains two atoms (Cu and Zn) per subunit and is commonly found as a tetramer in most organisms, although it can be sometimes be found as a dimer, and is present in extracellular fluids, including plasma, lymph and synovial fluid (Fattman *et al*., 2003; Nozik-Grayck *et al*., 2005).

When it comes to identifying SOD enzyme activity within an organism, both direct and indirect approaches have been developed. A number of different research studies have applied indirect approaches through the adoption of nitro blue tetrazolium (NBT) — a substance commonly applied in consideration of its convenience and simplicity of use. Notably, an indirect approach that makes use of NBT for the identification of superoxide radicals produced by hypoxanthine and xanthine through conversion into formazan dye is both common and convenient to apply.

All types of SOD (Cu/Zn, Mn and Fe) can be measured by the SOD assay. The assay is a simple, rapid and reproducible tool with which to measure activity in tissue homogenates, erythrocyte lysates, serum, cell lysates and plasma. The rate of reduction of the O_2 is linearly correlated with xanthine oxidase (XO) activity and indirectly correlated with SOD inhibition, so a colorimetric method can be used to determine the IC50 (50% inhibition activity of SOD or SOD- like materials).

Borković *et al.* (2011) investigated the feasibility of using antioxidant defense enzymes as biomarkers of oxidative stress in freshwater mussels (*Unio pictorum*). The catalase (CAT) and SOD enzymatic activities, in addition to the total protein concentration, and protein and SOD electrophoretic profiles, were examined in the digestive gland and gills of the freshwater bivalve *Unio pictorum* at two locations in the river Sava (Borković *et al.*, 2011). The results of the CAT and SOD activities in the freshwater bivalve reflected antioxidative and dissimilar metabolic activities (Borković *et al.*, 2011). Moreover, Box et al. (2008) determined the antioxidant enzyme response (glutathione peroxidase (GPX), CAT, SOD, the phase II detoxifying enzyme glutathione S-transferase (GST) and markers of oxidative damage, thioredoxin reductase (TR) and malondialdehyde (MDA)) in *Pinna nobilis*'s digestive gland and gill. They studied the antioxidant response effects in the bivalve colonised by the invasive macroalgae *Lophocladia lallemandii* (Box et al., 2008). All enzyme activities occurred in the digestive gland and gills, with CAT and SOD activities being higher in the gills than in the digestive gland (Box et al., 2008). On the other hand, GST activity and MDA levels were higher in the digestive gland (Box et al., 2008). The presence of *L. lallemandii* induced a significant increase in the antioxidant enzymes' activities in both the digestive gland and gills (Box et al., 2008), with the exception of the CAT activity in the gills. GST and TR activities were also increased in both tissues, as well as the MDA concentration (Box et al., 2008).

There are two main rationales when considering whether or not to measure SOD activity, which is known to provide an indication as to levels of oxidative stress in

exposed cockles' gills. Primarily, SOD manages uncontrolled radicals through conversion of superoxide anion ($O_2^{\bullet-}$) to H_2O_2 of a lower reactivity; this may be taken to infer that the enzyme has been dealing directly with such free radicals; therefore, the activity level of SOD can then be seen to mirror the actual oxidative stress levels identifiable in the exposed cells. Secondarily, other functions of enzymes are seen to be notably reliant on the SOD enzymes production of H_2O_2. The H_2O_2 generated by SOD is converted by catalase (CAT) into water (H_2O) and O_2; glutathione peroxidase (GPX) also transforms the H_2O_2 as generated by SOD into water through GSH (reduced glutathione) oxidation to GSSG (oxidized glutathione).

2.10.4 Thiobarbituric Acid Reactive Substances (TBARS)

Lipid peroxidation is recognised as an extremely valuable marker of oxidative stress, as higher amounts of TBARS (thiobarbituric acid-reactive substances) will be found in the biological cell membranes. There is an association between lipid peroxidation and the oxidative stress that is seen to arise when there is excessive generation of free radical oxygen species (Beltran *et al*., 2003; Oakes *et al*., 2003). As an example, in this regard, one well-characterised TBAR is malondialdehyde (MDA), which is recognised as an oxidation product of polyunsaturated fatty acids (PUFAs) (Camejo *et al*., 1998; Beltran *et al*., 2003). A greater rate of TBARS production may be recognised as a critical consideration in the context of diseases affecting the human population and is also highlighted as one of the causes underpinning various inflammatory lung disorders, including adult respiratory distress syndrome, bronchial

asthma, chronic obstructive pulmonary disease, and pneumonia, amongst others. In addition, TBARS may also be present in the lungs of asymptomatic cigarette smokers (Nowak *et al*., 2001; Beltran *et al*., 2003).

Since the 1950s, the application of MDA assay or TBARS has been significant in measuring lipid peroxidation levels in biological systems and membranes, and also to evaluate the degree of oxidation of polyunsaturated fatty acids (PUFA) through free radical-mediated means, in the case of both plant membrane and animal species (Du and Bramlage, 1992; Camejo *et al*., 1998; Hodges *et al*., 1999; Butterfield and Lauderback, 2002). Importantly the TBARS assay has become one of the most widely implemented approaches in the measurement of lipid peroxidation, offering a number of benefits, including affordability, simplicity, reliable results, sensitivity to minor TBARS changes in tissue, and requires minimal manipulation to assess a large number of samples (Hodges *et al*., 1999; Nowak *et al*., 2001; Schisterman *et al*., 2001; Almroth *et al*., 2005).

CHAPTER 3 MATERIALS AND METHODS

3.1 Introduction

This research aims to determine the bioavailability of SWCNTs and MWCNTs to the common cockle (*C. edule*), and their possible genotoxic effects, both individually and in combination with other common sediment-associated contaminants (cadmium and zinc), using a variety of predetermined concentrations and different exposure conditions. This chapter describes the processes used to select and collect the cockles, prepare the experimental tanks, dissect the cockles and extract the haemocytes and gills. It also describes how the chemical solutions and buffers were prepared for cell viability assessment (Trypan blue), as well as the processes of single cell gel electrophoresis (SCGE) or comet assay, cell viability, superoxide dismutase (SOD) and thiobarbituric acid reactive substances (TBARS). It explains which chemical concentrations were chosen and how they were prepared and the process of chemical analysis, as well as the instruments and equipment used and statistical analysis of the data.

3.2 Characterisation of Stock SWCNT and MWCNTs

To analyse the subject matter and correctly characterize the SWCNTs and MWCNTs, certain techniques and instruments were used: DLS, zeta potential, TEM and Raman

spectroscopy. Table 3.1 shows the properties of SWCNT and MWCNT powders used in this study.

Table 3.1: Properties of SWCNT and MWCNT powders. Data are based on those reported by the manufacturers.

Type	Manufacturer	Catalog No.	Purity	Diameter (nm)	Length (μm)	Density at 25 C°	Making method
SWCNTs	Sigma-Aldrich	704121	> 90%	0.83	1	1.7-1.9 g/cm^3	CVD
MWCNTs	Sigma-Aldrich	724769	> 95%	6 – 9	5	~2.1 g/mL	CVD

3.2.1 TEM (Transmission Electronic Microscopy)

TEM is a microscopy method which transmits a beam of electrons through a given specimen to form an image and can determine the structure and composition of different materials using diffraction patterns. First developed by Max Knoll and Ernst Ruska in 1931 (Freundlich, 1963), TEM can create images at a very high resolution (compared to light microscopes) due to the small wavelength of electrons. TEM was utilized here to evaluate the size and shape of CNTs at high resolution.

Firstly, the stock SWCNTs (1mg L^{-1}) were suspended in distilled water, using the dispersant 0.02% Suwannee River Natural Organic Matter (SRNOM) and treated in an ultrasonic bath (Decon FS300 Frequency Sweep) at 100 W, 80% pulse mode for 2 hours (Kobayashi *et al.*, 2009; Wu *et al.*, 2014). A small amount (1-2 drops) of stock suspension was pipetted onto a membrane grid, and a transmission electron microscope (TEM; Philips CM120-Biotwin) used to generate images (courtesy of Steve Mitchel, EM technician at the University of Edinburgh).

3.2.2 Dynamic light scattering (DLS) and Zeta potential

This technique is ideal for finding and evaluating clusters of NMs in various solutions, measuring the zeta potential (to deduce the surface charge) of NMs in solution, and for ascertaining the hydrodynamic size of NMs.

The surface charges of different SWCNTs and MWCNTs was derived from the zeta potential at pH 8.4. Concentrations of each SWCNT and MWCNT at 50μg L^{-1}, 100μg L^{-1}, and 500μg L^{-1} suspended in either seawater or distilled water. These suspensions were left for two hours in an ultrasonic bath reduce agglomerations formed during the preparation. DLA and zeta potential were derived from measurements taken with a red laser (Malvern Nano-ZS Zetasizer, Reference. No 2011143; wavelength of 633nm).

3.2.3 Raman Spectroscopy

Raman spectroscopy is a technique to detect and identify the rotational, vibrational and low frequency modes of molecules in a system. It uses the inelastic scattering from monochromatic light from a laser or suitable light source. Raman spectroscopy has capabilities to ascertain the localization of CNTs, to confirm their identity, diameter, their electronic behaviour, the number and structure of graphene layers' and whether there is any stress or strain in the graphene structure.

A quartz sample holder consisting of two quartz cover slips with a vinyl spacer in between was prepared. Quartz was utilised because it allows the lazer to reach the sample without any deviation. Onto this were placed samples of SWCNT stock and

MWCNT stock, which were then analysed using an inVia Raman spectroscope with an integrated confocal microscope with an operating laser at 785-nm and an output power of 5mW. The transmitted light was observed in two ways: the optical images of the stock SWCNTs and MWCNTs were captured using a 0.4 NA Leica N-plan microscope objective with a x20 magnification. Raman Spectroscopy (of the same area) was then performed using a 0.75 NA Leica N-plan at x50 magnification, where the lens objective was used to concentrate the laser's excitation beam through the CNTs. Using confocal laser scanning microscopy, the scattered light could then be observed and analysed and high resolution measurements taken in order to detect as many characteristic peaks (radial breathing mode, G band, G′ and D bands) of SWCNTs and MWCNTs as possible, using a wide range of Raman shifts (100–3200 cm^{-1}).

3.3 The Bioavailability of SWCNTs and MWCNTs to Sediment Dwelling Cockles.

3.3.1 Collection of Cockles

In this study, a total of 2,430 cockles (*C. edule*) were used as the sentinel and/or bioindicator organism for several reasons. Firstly, they were selected because their relative immobility can help to ascertain the area of pollution and the ability of cockles to accumulate contaminants from food and water, reflecting the bioavailable fraction; secondly, they are widely distributed, and their ease of collection makes them good

long-term biomarkers of environmental contamination (Figure 3.1). Thirdly, cockles are an essential part of the food chain, especially for mammals, and any infection which alters the cockle or their habitat, or interference by humans will have a direct effect on the life cycle of the cockle, and an indirect effect, by interrupting the food chain. *C. edule* are one of the most abundant bivalves found on sandy shores throughout northwestern Europe and are frequently exploited commercially (Sanchez-Salazar *et al.*, 1987).

Figure 3.1: Cockle Species in their Natural Habitats

3.3.2 Geographic range

A species distribution, or range, is the geographical area where a population of a biological taxon is spatially arranged. Key to this study, and in ecology and evolutionary biology in general, is analyzing and understanding of the forms that

geographic range limits of species take, and their causes and consequences (Gaston, 2009).

The specimens used for this thesis were of similar length (4 ± 0.4 cm) and collected from the causeway out to Cramond Island at the mouth of the river Almond, on the South coast of the Firth of Forth, East of South Queensferry (coordinates 55° 58' 04" North, 3° 18' 46" West) (Figure 3.2). This area is known for its high quality of water, which has greatly improved from industrial times and historical discharges (Scottish Environmental Protection Agency, 2006) and which has a known exposure history (Beanland, 1940). Cockles were collected by hand at low tide.

Figure 3.2: Location of Cramond Beach in Edinburgh

3.3.3 Aquarium seawater preparation

The collected cockles were immediately transported to the Heriot-Watt aquarium and kept in large tanks (50L). All tanks contained filtered and aerated seawater (15 ºC) (salinity: 32-34; T: 14ºC). They were left to acclimatize for at least 48 hours before use (Hartl *et al*., 2010; Figure 3.3). The tanks were checked regularly to assure no cockles were dead, and to change the seawater (twice a week). Cockles were fed algae (*Tetraselmis suecica*) three times a week, and the water and animal condition was checked daily.

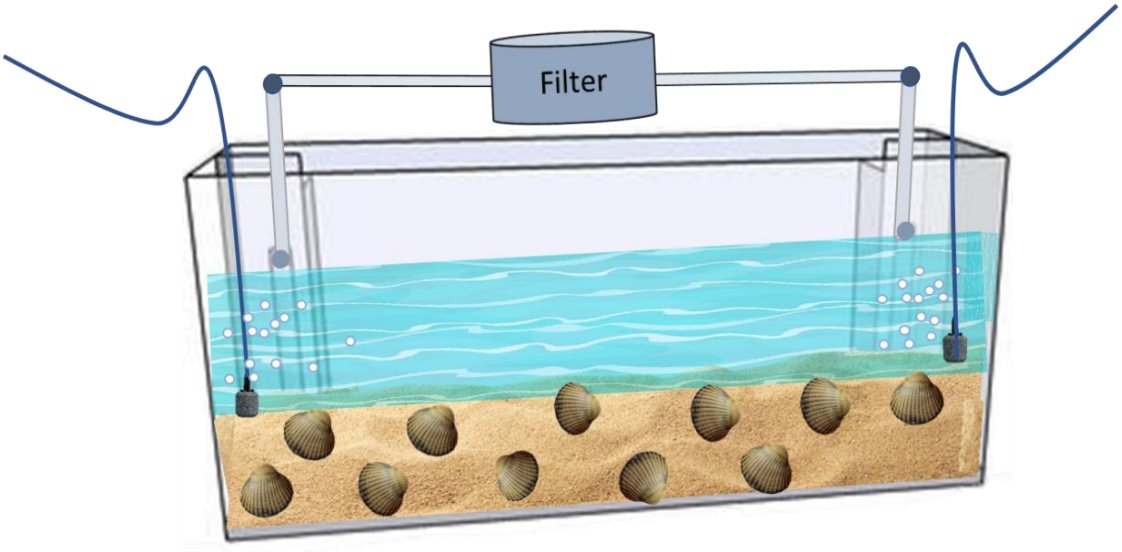


Figure 3.3: Holding Tank used to Store Cockles in the Aquarium Unit

3.3.4 Sediments

Two types of sediments were used in the present study: the first sediment type is the one that was used to hold the cockles in the aquarium, which corresponds to various habitats in which cockles are found (Figure 3.4 A). This sediment (type 1) was

collected from the coast at Cramond, Edinburgh, stored at 4°C to minimize consolidation effects and stirred bi-weekly to prevent hydrogen sulphide (H_2S) from building up. The second type (type 2) was a washed, lightly coloured, sediment purchased from "Bloom Sand" (Figure 3.4B). Sediment granulometry and total organic matter content were determined using a stacked sieve shaker for 15 minutes (Figure 3.4 C). The total organic matter was determined by loss of mass on ignition (Heiri *et al*., 2001).

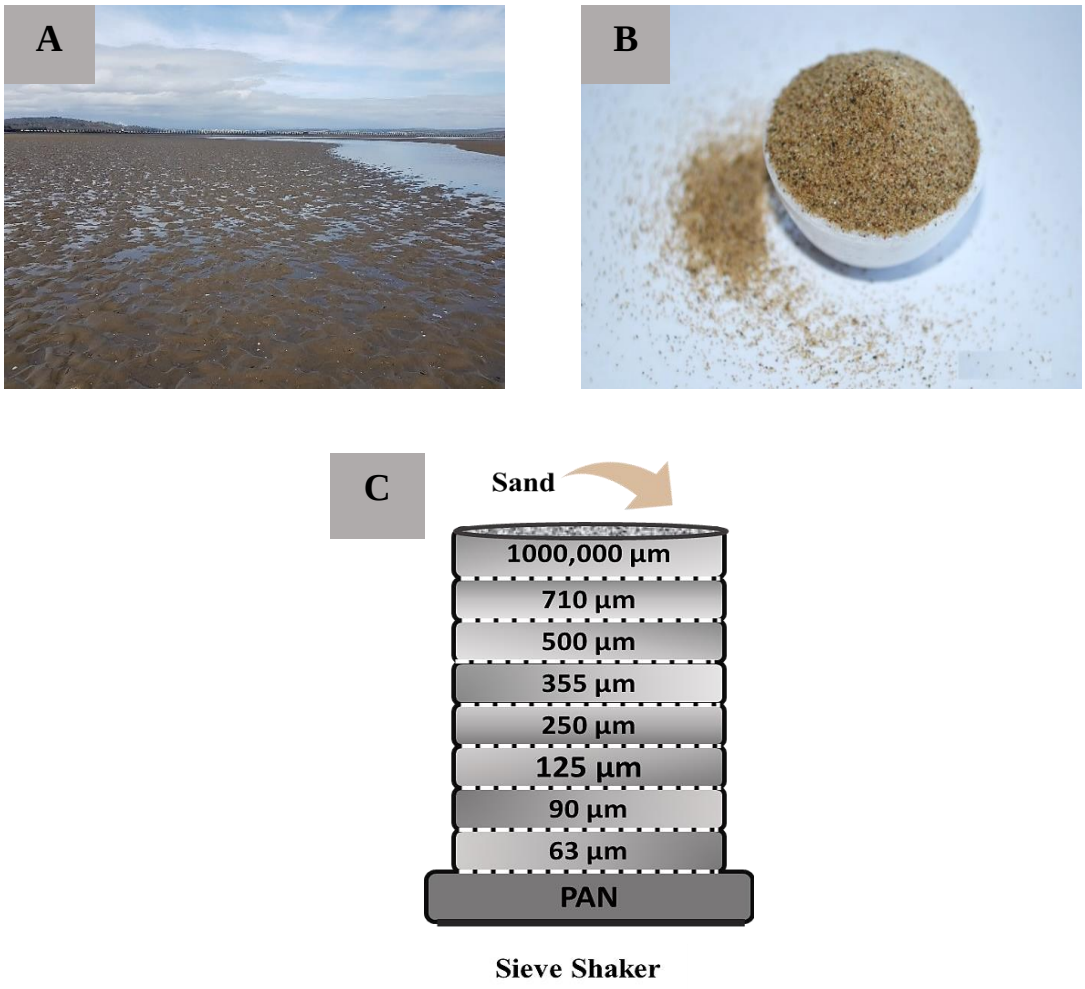


Figure 3.4: Sediments used. Natural Sediment (A), Purchased Sediments (B), and Sieve Shaker (C).

3.3.5 Bioavailability Exposure Condition

Three beakers (2L) were filled with 0.5 kg of washed type 2 sediments and 1 litre of seawater was carefully added and any re-suspended sediments left to settle. The water in each tank was then spiked with SRNOM-dispersed 100µg L^{-1} CNTs, either SWCNTs or MWCNTs. After that, three cockles (n=3) were introduced to each beaker. The first beaker was used as a control. The second beaker was used for treatment with SWCNTs, while the third beaker was used for treatment with MWCNTs. The concentration was chosen as an effective concentration, defined through preliminary experiments and was in line with previous work with mussels (Al-Shaeri *et al*., 2013), which was maintained for seventy-two hours. This experiment was repeated three times (n=9 in total) (Figure 3.5).

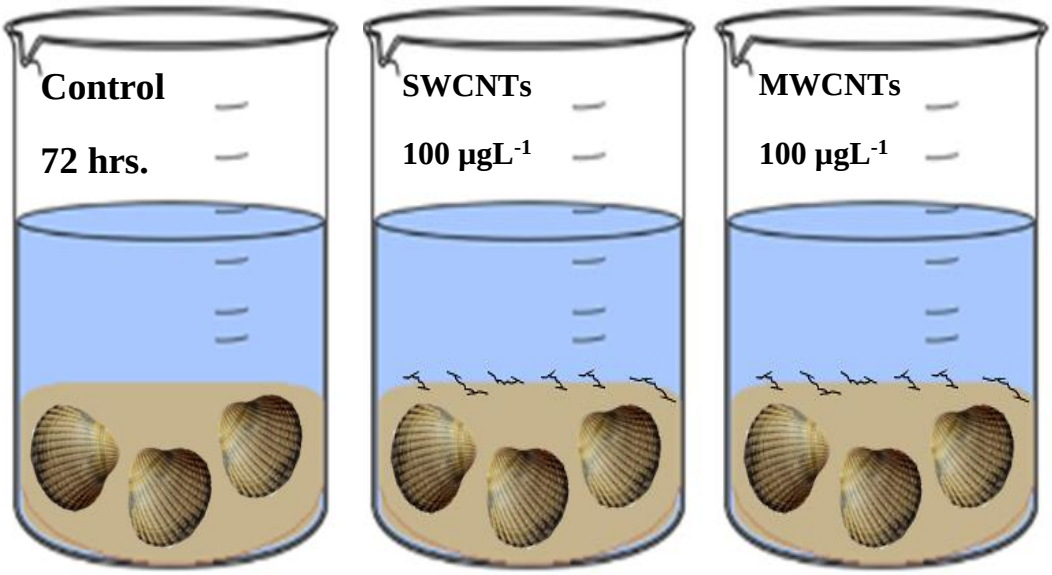


Figure 3.5: Control Tank and Treatment Tanks Spiked in vivo

3.3.6 CNT- Cockle Interaction

The aim of this experiment was to observe and assess extent of the interaction between the cockles and CNTs. It focused on cockle uptake of CNTs, and whether CNTs enter in to the cockle's tissues and cells. Cockled were exposed to CNTs, as described above. Once seventy-two hours had elapsed, the cockles were removed, sliced open by cutting the abductor muscles, and observed using a Leica MZ7s stereo microscope (x50 magnification), which was connected to a computer by a Leica DC300 digital camera to enable the images obtained to be captured (Figure 3.6).

Figure 3.6: Stereo microscope

3.3.6.1 Light Microscopy

Initially, this part of the study was designed to observe the adherence and agglomeration of CNTs to the gill and gut tissue of cockles using a light microscope.

Once the seventy-two hours of the bioavailability exposure **(see section 3.3.5)** period had elapsed, the cockles were removed from the tanks, and their tissue was extracted, dissected and histologically studied to determine whether the SWCNTs and MWCNTs had been ingested.

In this experiment, the cockles were positioned whole in clear, round, wide-mouthed jars. A small opening was made in the shell by lab scissors and the cockle was injected with Davidson's fixative: (20ml Formalin (40%), 30ml ethanol (100%)), 10ml of glacial acetic acid, along with 10ml of glycerol, and 30ml seawater.

The specimens, along with the fixative, were left for at least forty-eight hours, then the tissue was extracted in one piece from their shells using tweezers, and left in the fixative for an extra twenty-four hours, which ensured that all the tissues were fixed. Once this time had elapsed, the fixative was decanted and replaced with 70% ethanol for preservation (Kim and Hyun, 2006).

To prepare the tissues for processing, all tissues were transferred to labelled plastic cassettes, using the automatic Shandon Duplex Tissue Processor in a basket through the following: samples are carefully dehydrated with a graded absolute ethanol series: 70% ethanol for one hour (once), 90% ethanol for two hours, and 100% ethanol for

one hour (twice), and Histo-clear for three hours (once) (Stadtländer, 2007). Lastly, molten wax was added for 2hrs (2x) at 60-66ºC. In this dehydration process the cassettes are removed and replaced slowly, to dehydrate the tissues through exchange, using liquids which have a low surface tension and incorporating an ethanol series (Stadtländer, 2007).

To enable further examination, the tissues had to be embedded in paraffin blocks. This was achieved by pouring a little molten paraffin into a precast mould, adding the tissues using heated forceps, then transferring the mould onto a cold plate, where the tissue could be pressed into the paraffin, and lastly, more molten paraffin was added to cover the tissue. This was allowed to cool and harden for half an hour until the paraffin had set and the tissues could then be dissected. When the block was ready, it was sectioned using a rotary microtome (Ref: 577. LKB, Bromma, 2218 Historange microtome), with a new blade angled at 4°. The paraffin block was carefully sliced, and the very thin (10 µm thick) sections were placed in a water bath at 38°C, from where they could be placed onto slides for examination. The slides and sections were left to dry for one hour and then stained with an eosin/ haematoxylin method, and then covered with a 22x22 cover slip.

Finally, the gill and gut tissue slides of both the control and treatment samples were examined under a light microscope with total Magnification of (400x). This was connected to a colour camera (ZEISS Axiocam ERc 5s, 5 Megapixel), which was connected to a personal computer using ZEN 2.3 LITE software for image capture.

3.3.6.2 Raman Spectroscopy

3.3.6.2.1 Raman Spectroscopy for Cockle tissue

In this experiment, to be able to confirm material observed on gill epithelia as SWCNTs or MWCNTs, Raman spectroscopy (Figure 3.7) was employed.

Extraction of CNTs from cockle tissue

Once the time of bioavailability exposure condition had elapsed (see section 3.3.5), the cockles from all three tanks were removed, dissected, their gills extracted immersed at 4°C in a 2.5mL Hanks balanced salt solution (HBSS). The HBSS was prepared by adding 11.1g of sodium chloride (NaCl) to 500mL of HBSS ($22.2gL^{-1}$). In order to subject the gill tissues to Raman microspectrometrical analysis, tissue from all nine beakers was sectioned, and put into a quartz sample holder.

Using an inVia Raman spectrometer with an integrated microscope (Renishaw) operating with a power output of 5mW and with a 785nm laser attached, a Raman microspectroscopy was performed, on both control and the treatment samples. The transmitted light was captured using a 0.4 NA Leica N-plan microscope, which had a x20 magnification. Raman Spectroscopy (of the same area) was then performed using a 0.75 NA Leica N-plan at x50 magnification, where the lens objective was used to concentrate the laser's excitation beam through the CNTs.

The scattered light could then be observed and analysed, and high resolution measurements taken using confocal laser scanning microscopy. In order to detect as many characteristic peaks (radial breathing mode, G band, G′ and D bands) of SWCNTs and (D, G and G' bands) of MWCNTs as possible, a wide range of Raman shifts (100–3500 cm^{-1}) were counted.

Figure 3.7: Renishaw inVia Reflex Raman MicroSpectrometer equipment

3.3.6.2.2 Raman Spectroscopy for Sediment

In this study, in order to ascertain the spread of SWCNTs and MWCNTs into the sediment from the above experiments, Raman spectroscopy was used to determine and identify the presence of SWCNTs and MWCNTs in the spiked sediment /environment of the cockles.

Extraction of CNTs from Environmental Media

After seventy-two hours of bioavailability exposure (see section 3.3.5), core samples of the exposed sediment were taken randomly from three different areas of the sediment at the base of each beaker, using a cylinder, to a depth of 1 to 3 cm, which is generally the burial depth range of cockles. The sediment samples (~10 g) were transferred to a 200ml beaker. In order to change the density of the medium, the sediment samples were mixed with 100 ml of SDC 2% w/v and stirred for one minute at a speed of 3 rpm. The samples were then centrifuged 3000 rpm (2520 RCF) for 15 minutes, using a MSE Mistral 1000. The suspensions were then taken out by transfer pipette and filtered (pore size = 0.45µm, Whatman) (Figure 3.8), and placed under a Raman microscope spectrometer with an integrated Leica microscope ×50 (Figure 3.9).

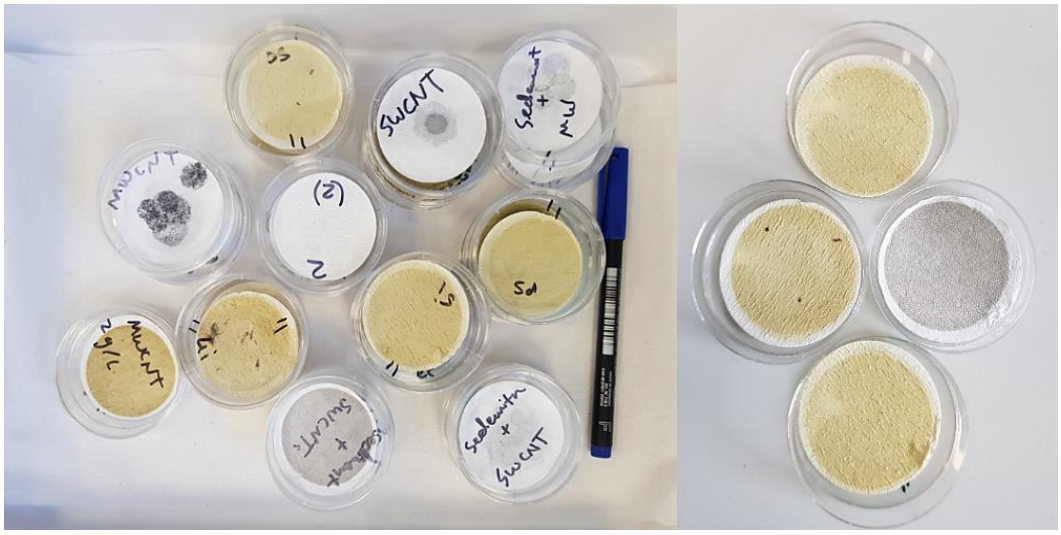


Figure 3.8: Filters of Exposed Sediments

After that, Raman mapping of the surface of the filter was carried out to detect the SWCNTs and MWCNTs in the sediment by raster scanning the Raman excitation beam over a 25 µm × 25 µm area of sediment. Raman spectra were acquired from multiple points on the sample, 1 µm apart (horizontal direction x), 1 µm apart (vertical direction, y) across the 2D surface in a process that took twenty-one hours. To detect the presence, if any, of SWCNTs, the intensity of the Raman shift at 1584 cm^{-1}, (the SWCNT G band) is plotted at each 1µm diameter pixel. Similarly, the intensity of the signal at a Raman shift of 1330 cm^{-1}, the (MWCNT D band) is plotted for MWCNTs (Figure 3.9).

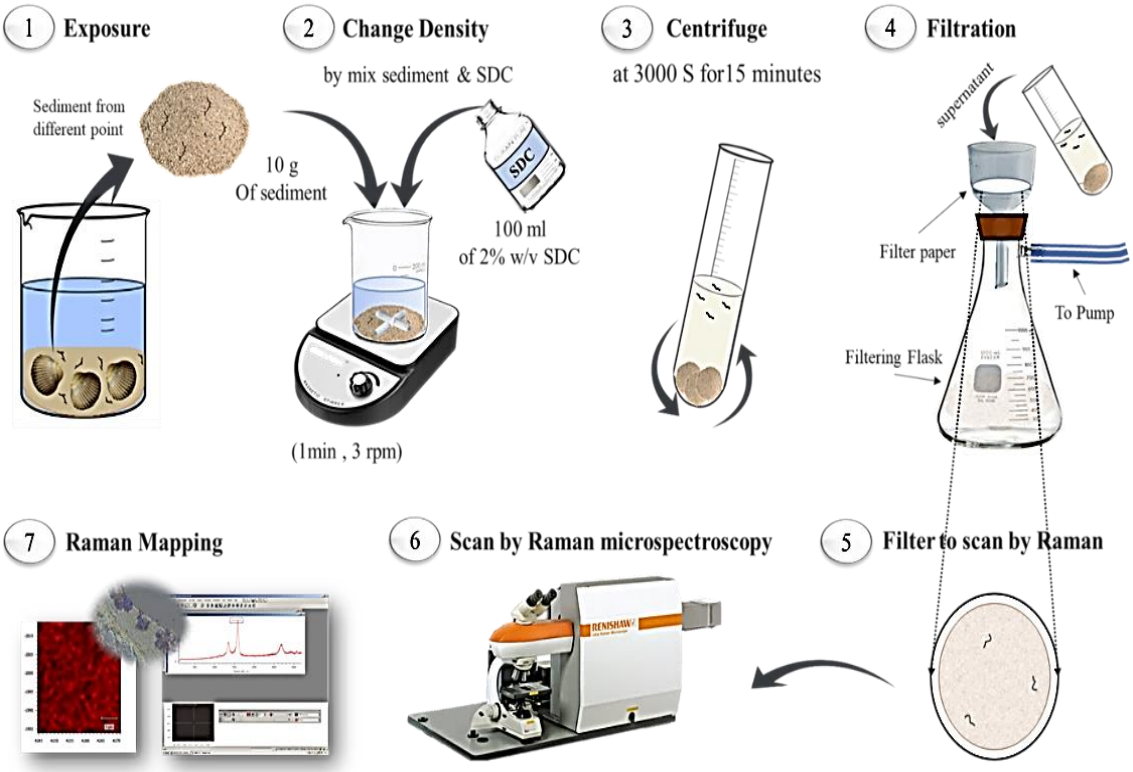


Figure 3.9: Raman Spectroscopy Overview Process for Exposed Sediment

3.3.6.3 Transmission Electronic Microscope (TEM)

Once the bioavailability exposure condition (see section 3.3.5) period had elapsed, the digestive gland and gills were extracted, sectioned and examined using a transmission electron microscope (TEM) to assess MWCNTs and SWCNTs internalisation in cockle cells (Figure 3.10A).

The sample was fixed in 0.1M sodium cacodylate buffer with 3% glutaraldehyde (with a pH of 7.3) for 120 minutes to improve fixation. The sample was then washed with 0.1M sodium cacodylate three times, every ten minutes. After washing, the samples were post-fixed, again in a 0.1M sodium cacodylate buffer, this time with 1% osmium tetroxide, for forty-five minutes. Then, using a 50%, 70%, 90% and 100% ethanol series, they were dehydrated for fifteen minutes, then in washed in propylene oxide to remove residual ethanol previously used for dehydration and finally pressed into a TAAB 812 resin. Using a Leica Ultracut ultramicrotome, the samples were sliced into ultra-thin sections, stained using Toluidine Blue, and examined to find an appropriate section to investigate using a light microscope. Once identified, the section was sliced very thinly (60nm) on the grid (Figure 3.10B), stained using uranyl acetate and lead citrate, and examined with a TEM (model JEOL JEM-1400 Plus), and each section was viewed with a GATAN OneView camera.

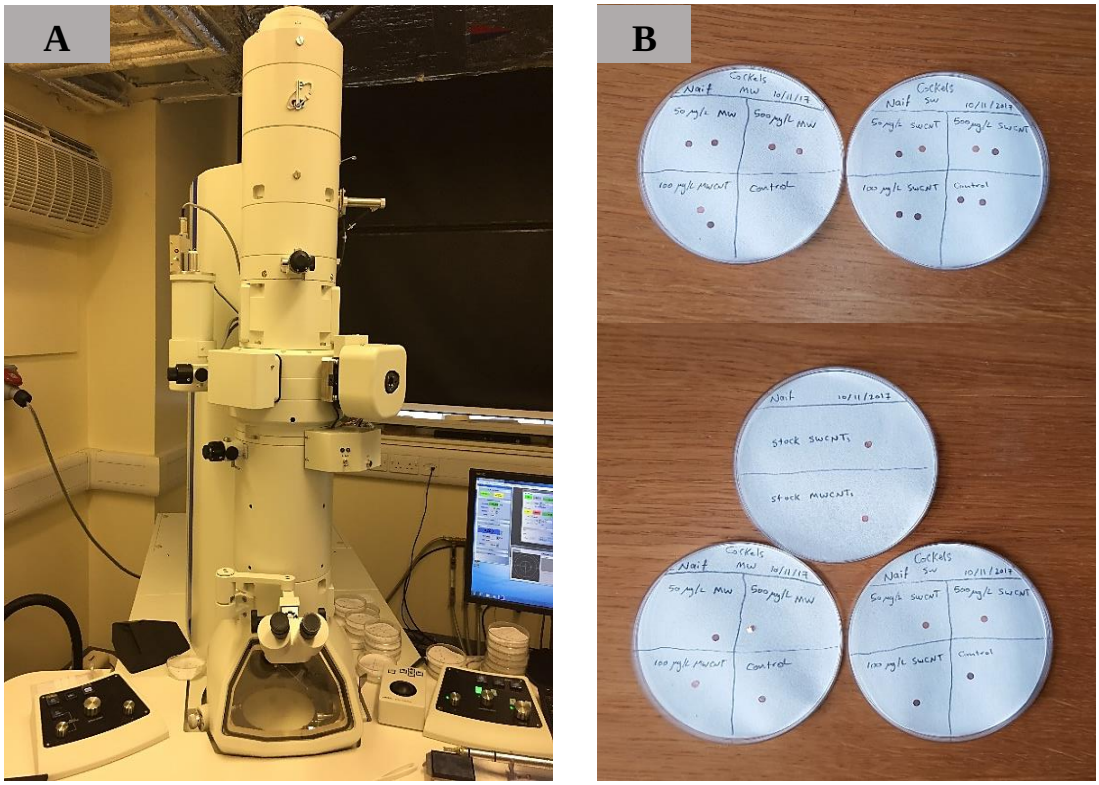


Figure 3.10: Transmission Electron Microscope (TEM) (A). CNT (SWCNTs and MWCNTs) samples on the grid (B). Transmission electron microscope samples were prepared with the help of Steve Mitchel (University of Edinburgh EM Unit).

3.4 The Toxicity of SWCNTs and MWCNTs to Sediment Dwelling Cockles

This experiment was designed to investigate the toxicity to cockles following exposure to SWCNTs and MWCNTs in the three different exposure treatments. The results were analysed to ascertain if the cockles have been affected by the CNTs using cell viability, comet assay and oxidative stress assays and in blood and gill cells.

The comet assay was used to determinate DNA damage, superoxide dismutase (SOD) was used to determinate oxidative stress and thiobarbituric acid reactive substances (TBARS) were used to determined lipid peroxidation. This experiment will address the question whether CNTs are more toxic in seawater, on the surface of the sediment or when the CNTs are mixed into the sediment.

3.4.1 Toxicology Exposure Conditions (Treatments)

This part of the study was aimed at determining the impact of the three treatment scenarios on the toxicity behaviour of MWCNT and SWCNT suspensions. In the first treatment (treatment 1), SWCNTs and MWCNTs were spiked into the water column; in the second (treatment 2), they were spiked onto the surface of the sediment, and in the third (treatment 3) they were mixed with the sediment (Figure 3.11). Three different concentrations were used to determinate the concentrations-response relationship. Separate negative and SNORM 0.02% control tanks were run in parallel. All the spiking described below was carried out in vivo for 72 hours, without changing

the water during that period to allow concentrate. All the experiments were replicated three times. (Figure 3.12, 3.13 and 3.14).

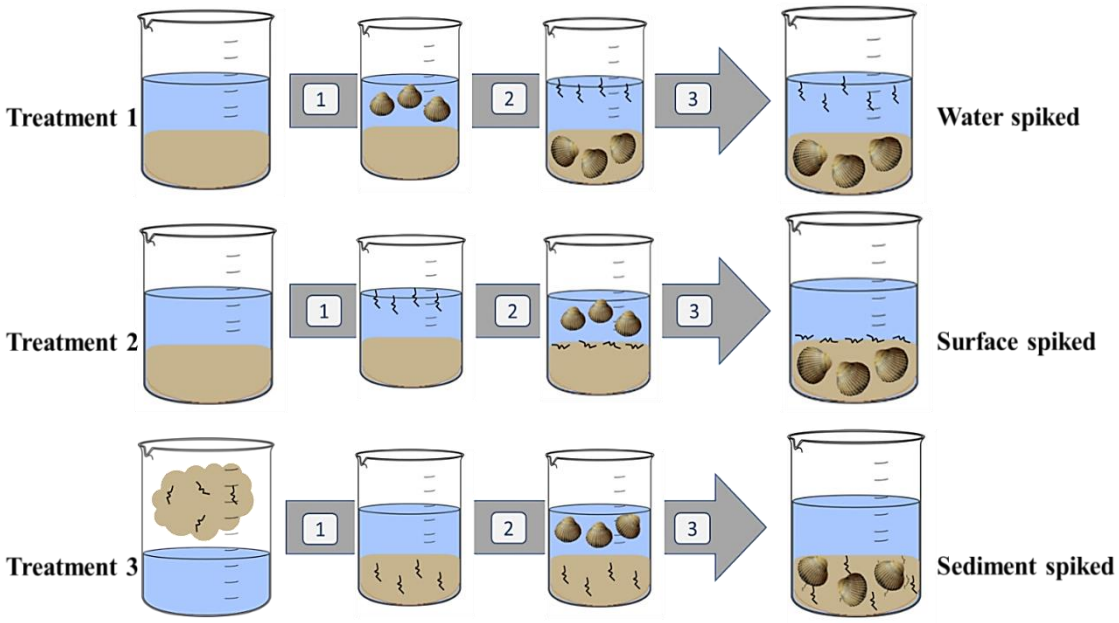


Figure 3.11: Experimental set-up for the three treatments of toxicological exposure conditions.

3.4.1.1 Treatment 1 (Water-spiked)

The aim of this treatment was to assess the time of settling down of CNTs onto the sediment and to confirm whether the cockles had absorbed the CNTs from the directly from the seawater medium. Thus, it aimed to confirm whether the spread of CNTs in the water column (rather than on the sediment surface or mixed into the sediment) would increase bioavailability and have a greater toxic effect on the organisms. In triplicate, 8x5L glass tanks were filled with 500g of washed sediment, 1L of seawater was carefully added, and any re-suspended sediment left to settle. Three healthy cockles were introduced into the system by placing them on to the sediment surface

and allowing them to bury themselves into the sediment. The water in each tank was then spiked with SRNOM-dispersed CNTs, (either SWCNTs or MWCNTs) at three-different concentrations: 50 μgL^{-1}; 100 μgL^{-1} and 500 μgL^{-1}, and left to settle onto the surface of the sediment and on the cockles (Figure 3.12).

The cockles were exposed in triplicate to:

Sample 1. Control

Sample 2. Suwannee River Natural Organic Matter (SRNOM).

Sample 3. SWCNTs 50 $\mu g\ L^{-1}$.

Sample 4. SWCNTs 100$\mu g\ L^{-1}$.

Sample 5. SWCNTs 500$\mu g\ L^{-1}$.

Sample 6. MWCNTs 50$\mu g\ L^{-1}$.

Sample 7. MWCNTs 100$\mu g\ L^{-1}$.

Sample 8. MWCNTs 500$\mu g\ L^{-1}$.

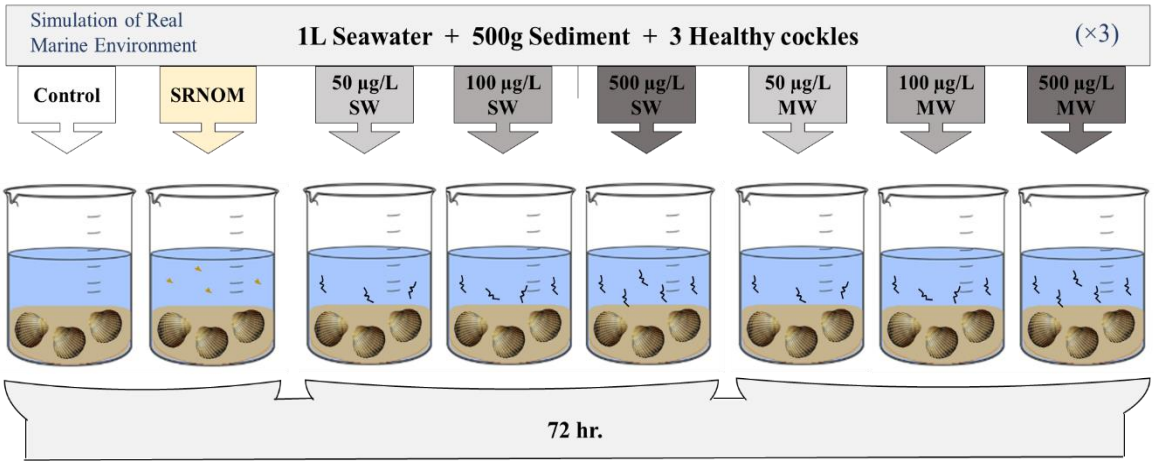


Figure 3.12: Treatment 1 Exposure Conditions and Concentrations used

3.4.1.2 Treatment 2 (Surface-Spiked)

The aim of this treatment was to observe the CNTs' behaviour and their bioavailability once they have settled on the sediment surface and to confirm whether the CNT bioavailability would increase relative to treatment 1. In triplicate, 8x5L glass tanks were filled with 500 g of washed sediment, 1L of seawater was carefully added and any re-suspended sediment left to settle. The water in each tank was then spiked with SRNOM-dispersed CNTs (either SWCNTs or MWCNTs), at three different concentrations: 50 μgL^{-1}; 100 μgL^{-1} and 500 μgL^{-1} and left to fully settle onto the surface of the sediment for 24 h before adding the cockles. Healthy cockles were introduced into the system by placing them on to the sediment surface and were allowed to bury themselves in the sediment. (Figure 3.13).

The cockles were exposed in triplicate to:

Sample 1. Control

Sample 2. Suwannee River Natural Organic Matter (SRNOM).

Sample 3. SWCNTs 50 $\mu g\ L^{-1}$.

Sample 4. SWCNTs 100$\mu g\ L^{-1}$.

Sample 5. SWCNTs 500$\mu g\ L^{-1}$.

Sample 6. MWCNTs 50$\mu g\ L^{-1}$.

Sample 7. MWCNTs 100$\mu g\ L^{-1}$.

Sample 8. MWCNTs 500$\mu g\ L^{-1}$.

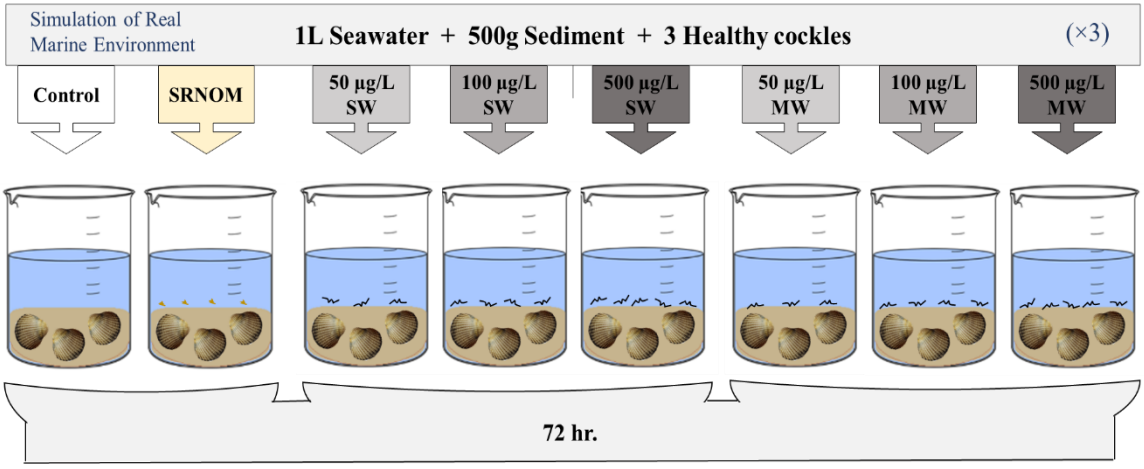


Figure 3.13: Treatment 2 exposure conditions and concentrations used

3.4.1.3 Treatment 3 (Sediment-Spiked)

The aim of this treatment was to observe behaviour and bioavailability of CNTs when mixed into the sediment, and how this affected the toxicity towards exposed cockles.

Glass tanks were prepared as described above. However, in this experiment, the SRNOM-dispersed CNTs were mixed into the sediment in a Thermo-MAXQ 3000 shaker for five minutes at 200 RPM before adding seawater, to give the following nominal sediment concentrations, which were equivalent concentrations to treatment one of the CNTs: 0.1 µg,g^{-1}, 0.2 µg.g^{-1} and 1 µg.g^{-1}. Seawater was added gently, to avoid excessive resuspension of sediment, which was left to settle; the cockles were then added as described above (Figure 3.14).

The cockles were exposed in triplicate to:

Sample 1. Control

Sample 2. Suwannee River Natural Organic Matter (SRNOM).

Sample 3. SWCNTs 0.1 $\mu g.g^{-1}$.

Sample 4. SWCNTs 0.2 $\mu g.g^{-1}$.

Sample 5. SWCNTs 1 $\mu g.g^{-1}$.

Sample 6. MWCNTs 0.1 $\mu g.g^{-1}$.

Sample 7. MWCNTs 0.2 $\mu g.g^{-1}$.

Sample 8. MWCNTs 1 $\mu g.g^{-1}$.

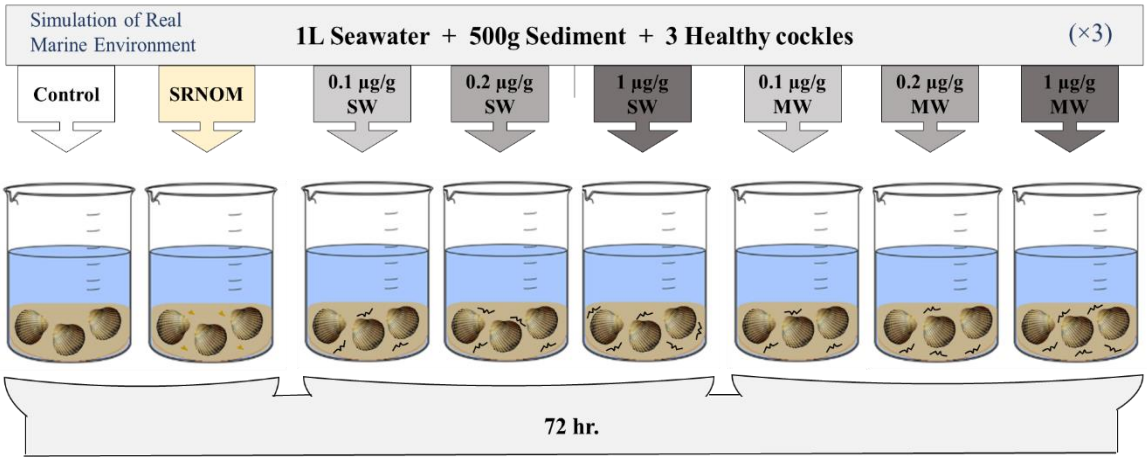


Figure 3.14: Treatment 3 exposure conditions and concentrations used

3.4.2 Biomarker Analysis

Biomarker analysis is used in many different fields of science as a quantifiable approach to assess a biological condition or state. This experiment aimed to assess the potential genotoxicity of CNTs, SWCNTs and MWCNTs, both in combination and separately, and how they may affect the cockles' cell viability, DNA damage and oxidative stress. The endpoints assessed were superoxide dismutase (SOD), cell viability (Trypan blue), thiobarbituric acid reactive substances (TBARS) and the comet assay or single cell gel electrophoresis.

3.4.2.1 Cell Isolation

After exposure to the three treatments, the cockles were prepared using the procedure described by Coughlan *et al*., (2002) for the clam *Tapes semidecusatus*, which was adapted for mussels by Singh and Hartl (2012). Using a 1ml Plastic Luer syringe (Ref: No. 300013) (21G1 needles; Sigma-Aldrich), scissors, a scalpel blade and tweezers, the cockles were dissected in order to isolate hæmocytes and gill cells.

3.4.2.1.1 Haemocyte Sample Extraction

To prepare the cockles for extraction of the hæmocytes, they were first placed with the bivalve facing down to facilitate dissection. The shells were prised apart using scissors, and any excess fluid allowed to drain out. To prepare the cells for removal with a syringe, first the syringe had to be prepared to prevent damage to cell tissue or death of the cell after extraction, which was achieved by using a Hanks' Balanced Salt

Solution (HBSS) from Invitrogen (Reference. No.14175-053 500mL; Ca and Mg-free): HBSS is a salt medium high in bicarbonate ions, originally formulated by John H Hanks in 1940, and used as a buffer to help maintain an optimum pH for a cell's growth after extraction from the sample. Since the hæmocytes and gill cells of cockles have an osmotic concentration of 990 mOsmol L^{-1}, the HBSS had to be altered to reflect this, so 22.2g L^{-1} of sodium chloride (NaCl) (from Sigma Aldrich) was added (Hartl *et al*., 2010). The needle was gently removed from the syringe, which contained a mixture of 0.1ml HBSS inside, was presented to the posterior abductor muscle of the cockle, and 0.1ml of the hæmocyte, and the 0.2ml mixture was transferred to a 1.5 ml Eppendorf tube (purchased from Greinerbio-one) and kept on ice (Figure 3.15).

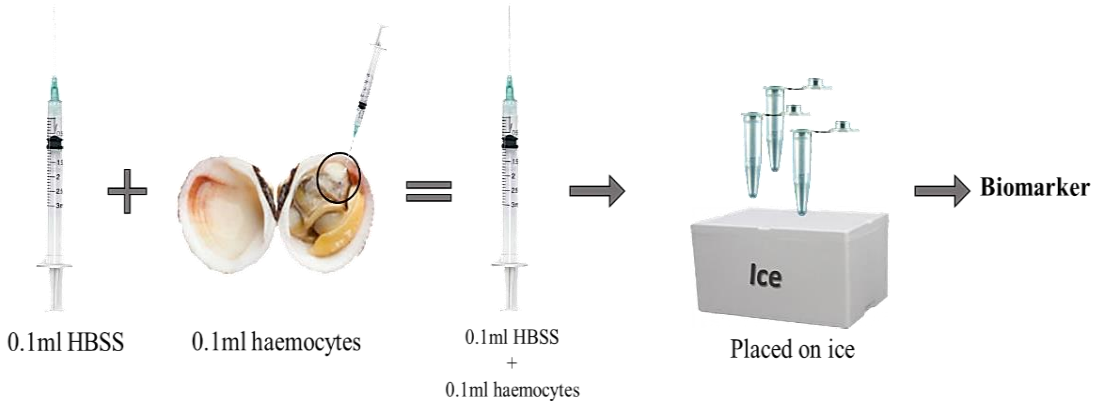


Figure 3.15: Haemocyte Isolation Procedure

3.4.2.1.2 Extraction of Cockle Gill Samples

The gill extraction was carried out using an adaption of a procedure utilized by Coughlan *et al*. (2002), (summarised in Figure 3.16). Initially, a 2.5% trypsin solution had been prepared (10x from porcine pancreas was purchased from SAFC stored at -

20°C). Using a fresh scalpel, the cockles were dissected, opened up and the gills extracted. The gills were then isolated and placed in Petri dishes which had 2.5ml salt buffer (HBSS) and altered osmotically with 22.2g L^{-1} of sodium chloride (NaCl). Using fresh scalpel blades with a scissor-like motion, the gills were sliced ten times and then transferred, using wide-mouthed Pasteur pipettes and, still in the salt buffer, to (PP-Test tubes, 15mL) test tubes containing 2.5 ml 0.1% buffered trypsin solution, to give a final enzyme concentration of 0.05%. The tubes then gently rocked on a gyro-rocker (SSL3) for ten minutes at room temperature. Once this was completed, HBSS was added to the tubes to obtain a uniform 10ml volume and dilute the trypsin. The HBSS/gill cell solution was then filtered using a 40µm cell strainer, then centrifuged for ten minutes at 3000rpm with a MSE Mistral 1000 to separate the pellets from the supernatant, which were then extracted, and added to 0.5ml of HBSS (trypsin free) for re-suspension.

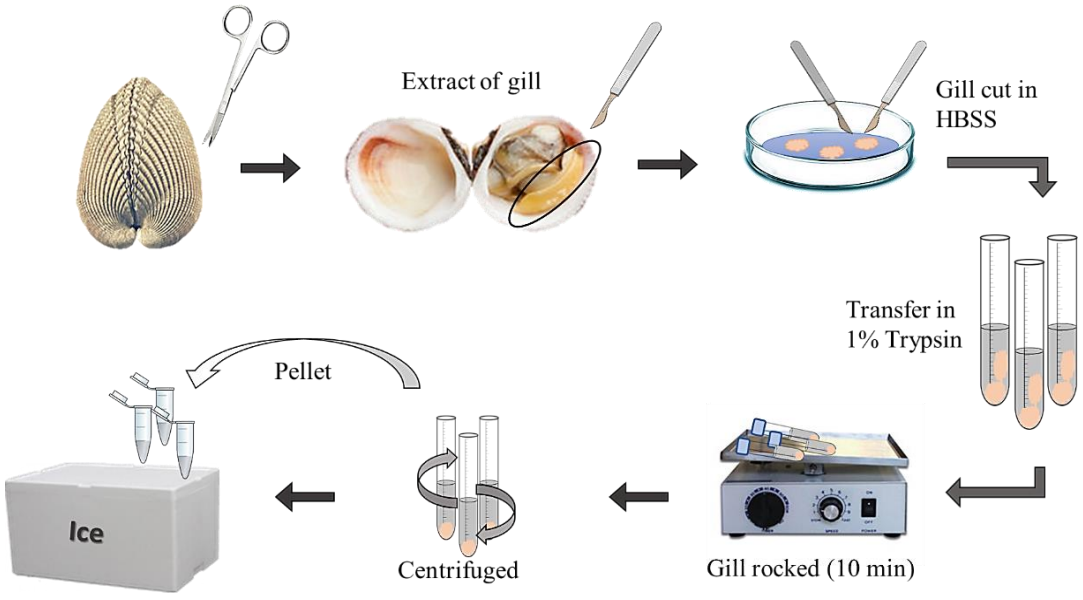


Figure 3.16: Gill Isolation Procedure

3.4.2.2 Cell Viability Using Trypan Blue

In order to test cockle hæmocytes for damage caused following exposure to different forms of CNTs at different concentrations, cell viability in both the exposed and control samples must be ascertained. This was achieved by using Trypan blue which is a straightforward and simple technique used to stain the dead cells which can be seen with the naked eye and counted within 100 cells under a light microscope.

Following Absolom (1986), a trypan blue assay was prepared; this was prepared on the day by mixing four parts (80µL) of trypan blue dye 0.2% (w/v) with one part (20µL) of sodium chloride (NaCl) 4.25% (w/v) in water. Subsequently, trypan blue stain solution was mixed with100µL of haemolymph cells, which were accordingly pipetted onto a haemocytometer (Neubauer Improved Haemocytometer) which had a special cover slip (22mm X 22mm) carefully placed on top (Figure 3.17). The suspension fills the counting chamber (Figure 3.18A) by capillary action, and as the counting chamber is a known depth and volume, the cells can be counted using a light microscope (Figure 3.18B).

3.4.2.2.1 Exposure to Treatment

Once the cockles had been exposed under toxicology exposure conditions for seventy-two hours **(see section 3.4.1),** they were removed from the tanks, and their haemocytes and gill cells extracted by dissection. These cells were placed in Eppendorf tubes and placed on ice. Cell viability was then ascertained using a trypan blue dye as before.

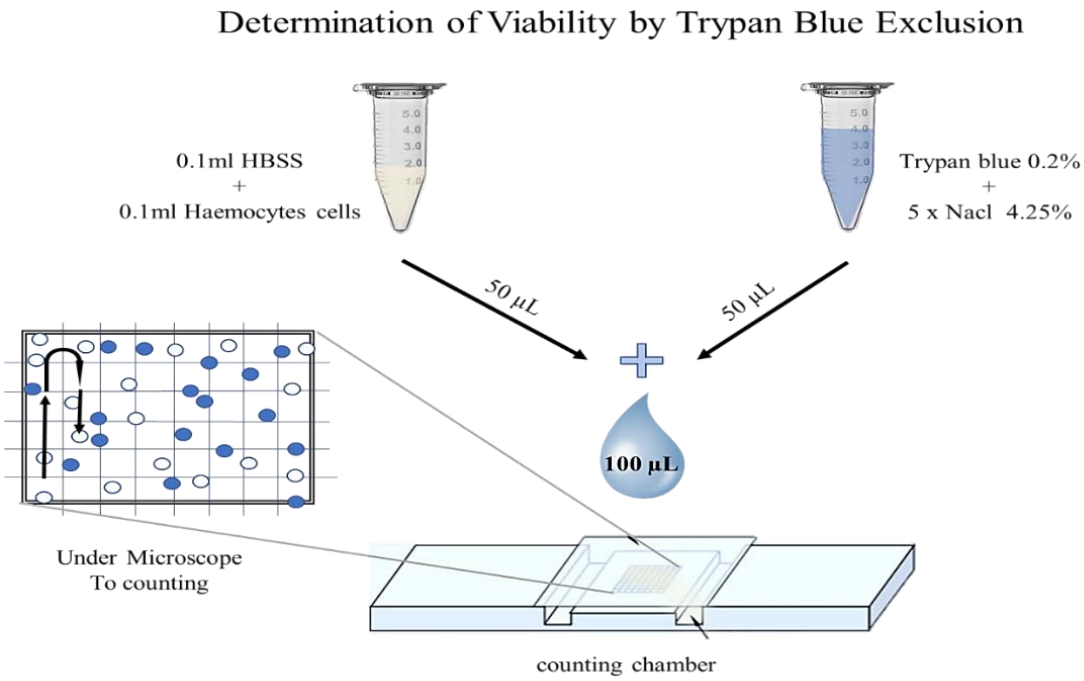


Figure 3.17: The Trypan Blue Assay Procedure

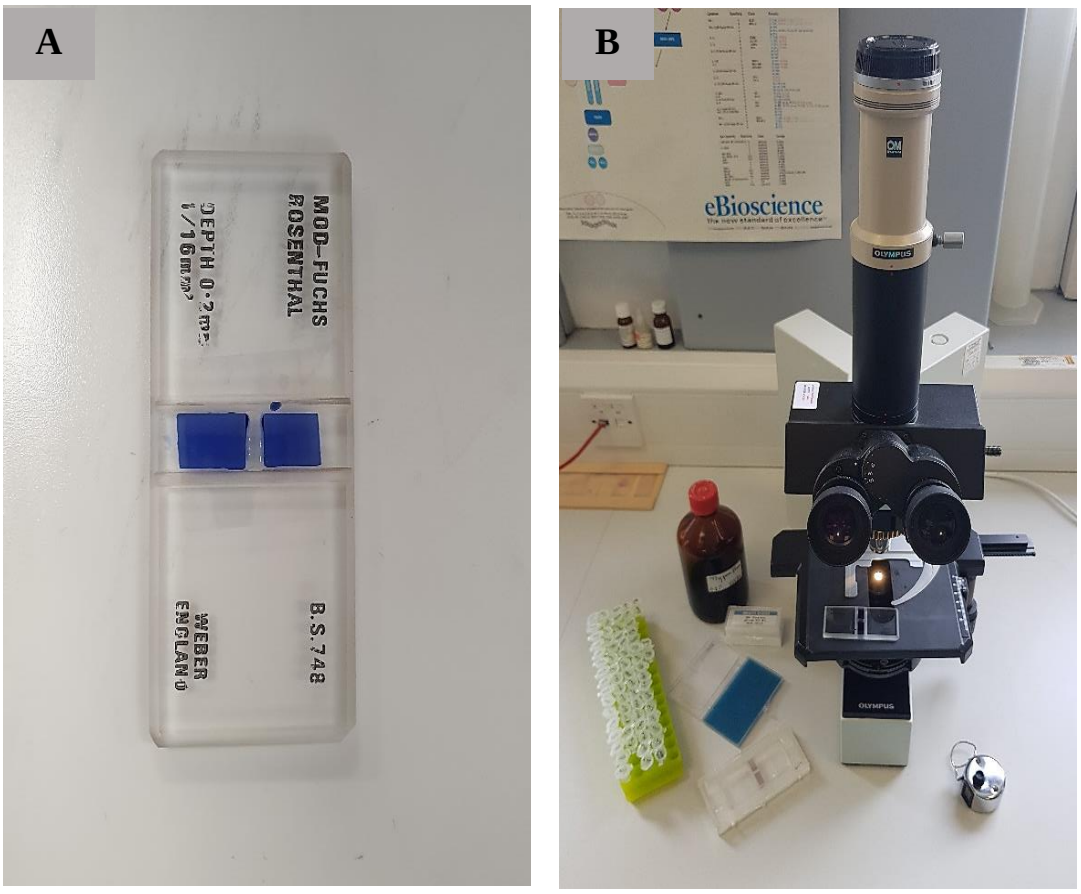


Figure 3.18: The Trypan Blue Slide (Counting Chamber) (A), The Light Microscope Counter (B).

3.4.2.3 Comet Assay

A comet assay was performed, using the same procedure as for the clam, *Tapes semidecusatus* described by Coughlan *et al*. (2002), which was adapted for mussels by Hartl *et al*. (2010). This assay was carried out to ascertain how the genotoxicity of SWCNTs and MWCNTs will affect the DNA of a cockle's haemocytes and gill cells.

As a minor change here, GelRed (Biotum, catalogue BT41003; solution 2 μL in 10 mL), a fluorescent DNA stain, replaced ethidium bromide (summarised in Figure 3.19).

3.4.2.3.1 Exposure to treatments

1 L of filtered, aerated seawater (with a salinity range of 33±1 ppt, at 14 °C) was placed in each of eight glass beakers with 500g of sediment and fitted with aquarium air pumps. Three cockles (length 3-4 cm) were added to each mixture. The cockles were exposed in triplicate in the three treatments differently as described in **section 3.4.1**

Experimental Chemicals, Stains, and Reagents Used

Following the protocol described by Hartl *et al*. (2007), solutions were prepared thus:

Phosphate Buffered Saline (PBS)

One tablet from Sigma-Aldrich (PBS Reference: 096K8217) was added to 200ml of distilled water and maintained at 25ºC.

Normal Gel Agarose (NGA)

Using a 100ml conical flask, 1% (1 gram) of Normal Gel Agarose NGA from Sigma-Aldrich (Agarose, Type V; Reference: 08K1093) was dissolved in a PBS solution, and maintained at 4°C.

Low Melting Agarose (LMP)

Using a 100ml conical flask filled with 1 gram of PBS solution, 1% of low melting point Agarose from Sigma-Aldrich (Agarose, Type I-B; Ref: 075K0077) was dissolved and maintained at 4°C.

Sodium Hydroxide (NaOH)

400 g of NaOH from Sigma-Aldrich (M.M 40, Ref: 28248.367) was added to 1000ml of distilled water and dissolved.

Ethylenediaminetetra-Acetic Acid (EDTA)

23.37g EDTA from Sigma-Aldrich (ACS reagent, 99.4-100.06%; Ref: 028k00581) was added to 0.2L of distilled water, and the mixture brought to pH7 using NaOH. An additional 200ml of water was poured in to bring the volume up to 400ml.

Tris Hydrochloride (Tris)

31.52g of Tris ($C_4H_{11}NO_3$ CIH) from VWR International Ltd (Ref: 441514A) was added to 0.5L of distilled water and dissolved.

Lysing Solution Stock

In a beaker of 1.5L of distilled water, 100mM (44 g) of EDTA and 2.5M (84.6 g) of NaCl were added and dissolved, then 10mM (15 g) of Tris hydrochloride poured in. To bring the pH up to 10, NaOH was slowly added.

Lysis Working Solution

To obtain this, 135ml of the previously prepared lysis solution was poured into a small black tank and 15ml of dimethyl sulphoxide (DMSO) and 1% (1.5ml) Triton X-100 from Sigma-Aldrich (ref: BCBB4025 and 22-41-51/53 respectively) were added to this). This solution was made up fresh every day and maintained at 4°C in the dark until use.

Electrophoresis Solution

2L of chilled, distilled water was prepared, and 10ml of 200mM EDTA and 60ml of 10N NaOH were mixed. Final concentrations were 1mM EDTA and 300mM NaOH. This solution was stored at 4°C.

GelRed Stain

A 2μL of GelRed (Biotum; ref: BT41003) was diluted in 10mL of water to produce the GelRed working solution.

Procedure for Comet Assay:

Initially, a group of microscope slides (0.1-1.2mm from Fisherbrand, ref: FB58628) was prepared. After marking with a pencil for orientation, 1% Normal Gel Agarose (NGA) was heated up and 100μL pipetted onto each slide and spread evenly to encourage frosting. The slides were then air dried for twenty-four hours.

First Layer

The previously prepared microscopic slides were heated up using a hot plate (at 40°C) to eradicate any air pockets and to form an even spread of the NGA. Then 100 μL of previously prepared NGA was pipetted directly onto the slide, and a 22mmx22mm microscope cover slip (Thermo Scientific) was carefully placed on top. To expedite the cooling of the gel, the slides were stored on ice for 15-20 minutes.

Second Layer

After the gel had cooled, and the cover slips gently removed, the second layer was prepared. A mixture was formed by melting a Low Melting Point (LMP) agarose gel, which was then mixed with either haemocytes or a gill cell suspension, and this was pipetted onto the slides. Three different volumes (30µl, 70µl and 100µl) of LMP were carefully pipetted out: firstly, 30µl of a suspension of gill cells or haemocytes were drawn and added to an Eppendorf tube; secondly, in the same Eppendorf ,70µl of low melting point gel was added; thirdly, 100µl of a suspension of composite LMP and gill cells or haemocytes were pipetted out and placed on the slide, a 22x22mm cover slip placed on top, and then left for twenty minutes at 4°C.

Third Layer

Once this had set, 100µL of LMP was drawn out and pipetted onto each of the slides as per the first layer (this time the NGA was replaced with LMP). Again, each slide was covered by a 22mmx22mm microscope slip, and cooled to 4°C.

Lysis

After cooling, the cover slips were carefully removed. To check for single strand breaks, the cell membrane was removed using an alkaline lysis solution which had been prepared earlier (see above). The slides were placed in this solution, and stored in a cold, unlit room at 4°C, which will minimize DNA damage. As Kantor and Barnhart (1973) showed, DNA strands can be formed by light (UV radiation), causing pyrimidine dimers, which create DNA fragments and would consequently alter the

count and give a false reading. Some gill cells were separated and frozen in liquid nitrogen, at -80ºC for an oxidative stress assay, **described later (3.4.2.4).**

Electrophoresis Buffer and Unwinding

In order to enable denaturing and unwinding of the DNA so that the single-strands DNA breakes (SSBs) could be counted, the alkaline solution (300mM L^{-1} NaOH and 1mM L^{-1} EDTA, pH 10, as pH >13 cause disassociation of alkali labile sites, which would inflate the SSB counts) was added to 2L of distilled water. The slides, spaced randomly but with the pencil marks showing upwards, were placed horizontally, left to right on an electrophoresis plate (from Owl Separation Systems, Inc., Rated 0-250V, 0-100mA, Model A3-1, Serial No. 233712), and the alkaline solution poured onto the electrophoresis plate to immerse the slides. The plate was left for 30 mins to allow the DNA to unwind, then the power and current were turned on and adjusted to 250V with 300mA and left for exactly 25 mins in the dark at 4°C. This has been applied in order to gain the desired the disassociation of DNA.

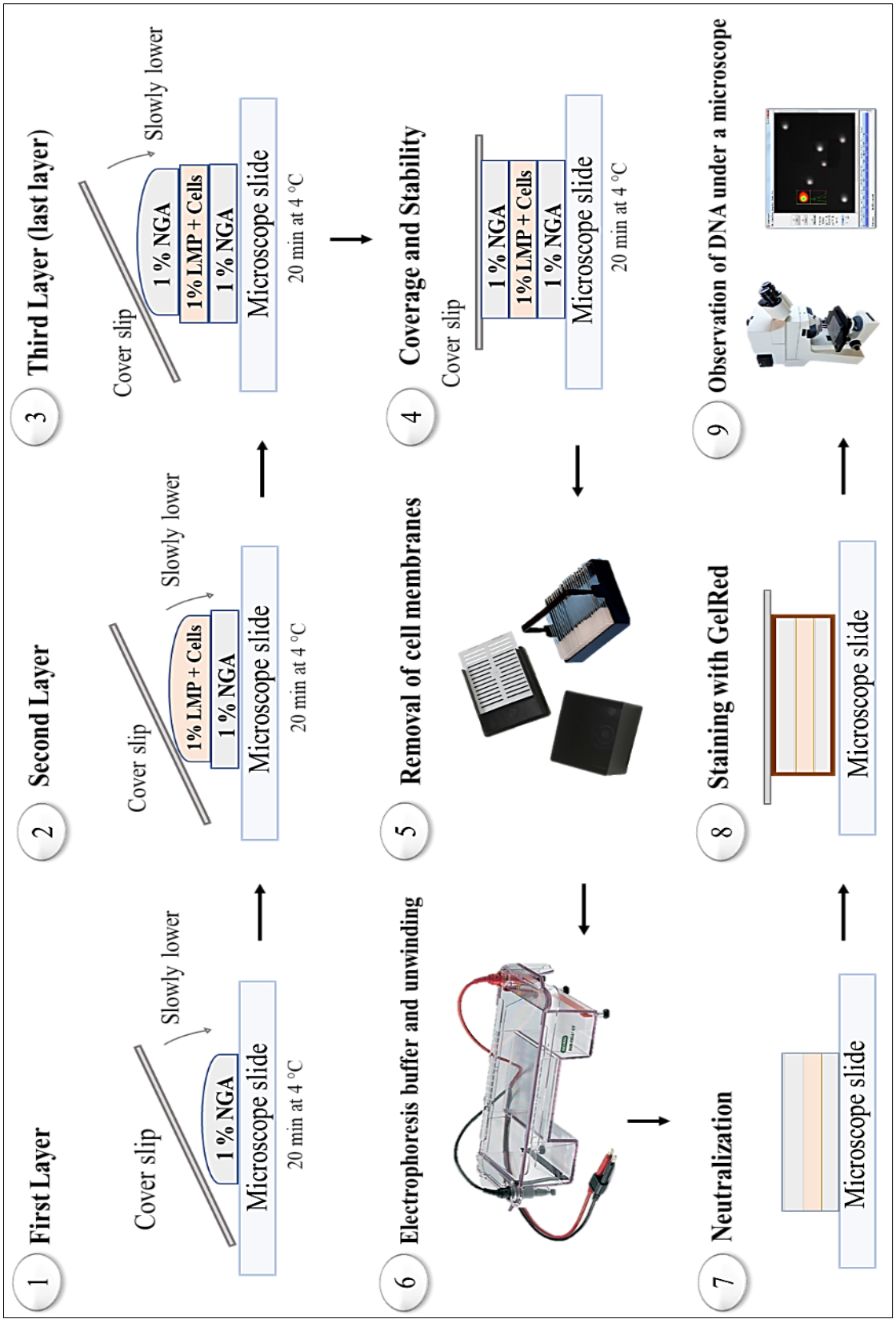


Figure 3.19: Summary of the Comet Assay Procedure

Neutralization and Staining

Once the electrophoresis time period had elapsed, the slides were taken from the electrophoresis tank for gel neutralization in order for the GelRed to bind properly.

A pipette was prepared with 0.4 M Tris (pH 7.5) and five drops were placed on to the slides; this was repeated 3 times followed by a rinse with five drops of chilled distilled H_2O. Excess fluid was removed by tilting the slides, and the slides were stained with GelRed. Cover slips were then carefully placed on the slides, which were then placed in humid boxes at a temperature of 4°C, ready to be scored.

Using a Microscope to Observe Single Strand DNA Breaks

Following neutralization and staining, the slides, still with their cover slips, were placed in the stage clips of a Zeiss Axiophot microscope (Reference No. 58) with the objective magnification being adjusted to ph_2-plan-Neofluar 40x/0.75 (∞/0.17), and an ocular lens setting of Pl 10x/25, a suitable magnification for counting cells and detecting single strand DNA breaks. Secondly, employing a Comet Assay IV (from Perceptive Instruments) with live video scoring, fifty cells from each slide were studied to measure the % DNA tail which was expressed as the average of 50 measurements. IV software shows cellular DNA damaged (containing fragments and strand breaks) which separated from intact DNA. The Traditional "comet tail" shape has been shown under the microscope (Figure 3.20).

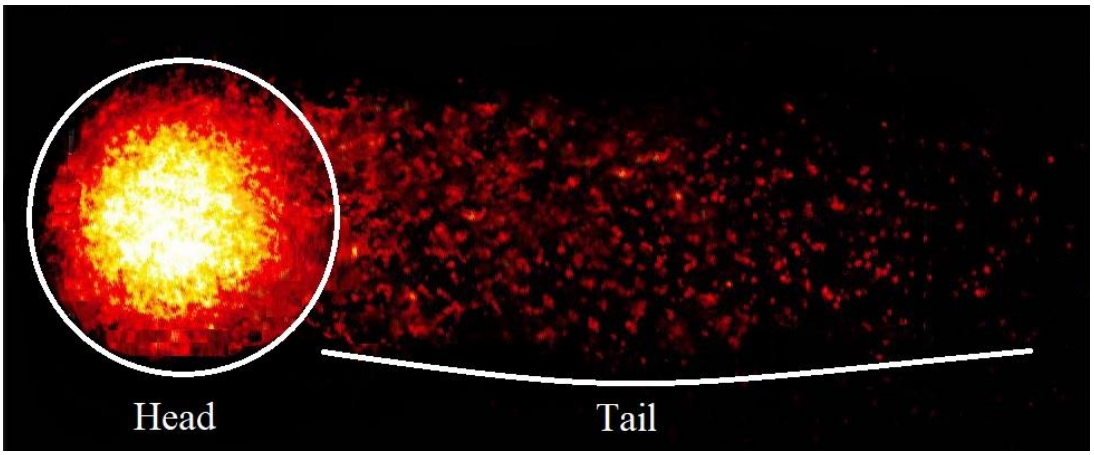


Figure 3.20: DNA damage image when analyzed by comet assay IV software

3.4.2.4 Oxidative Stress Assays

During gill cell membrane isolation for the comet assay, the part of the gills excised previously and frozen in liquid nitrogen and stored at –80 °C for a day were then carefully homogenized on ice, with 750 µL of buffer (tissue buffer = 1:5) (Tris-HCl 50 mM, 0.15M KCl, pH 7.4), and then centrifuged for twenty minutes (at 10,000g and 4°C). The resulting pellet was kept to be analysed using TBARS assay. The remaining supernatant fraction was taken and placed in an ultracentrifuge tube (Beckman, 5ml), whereupon it was centrifuged for sixty minutes (at 40,000g and 4°C) to produce the cytosolic fraction. This fraction could then be analysed for SOD activity (Figure 3.21).

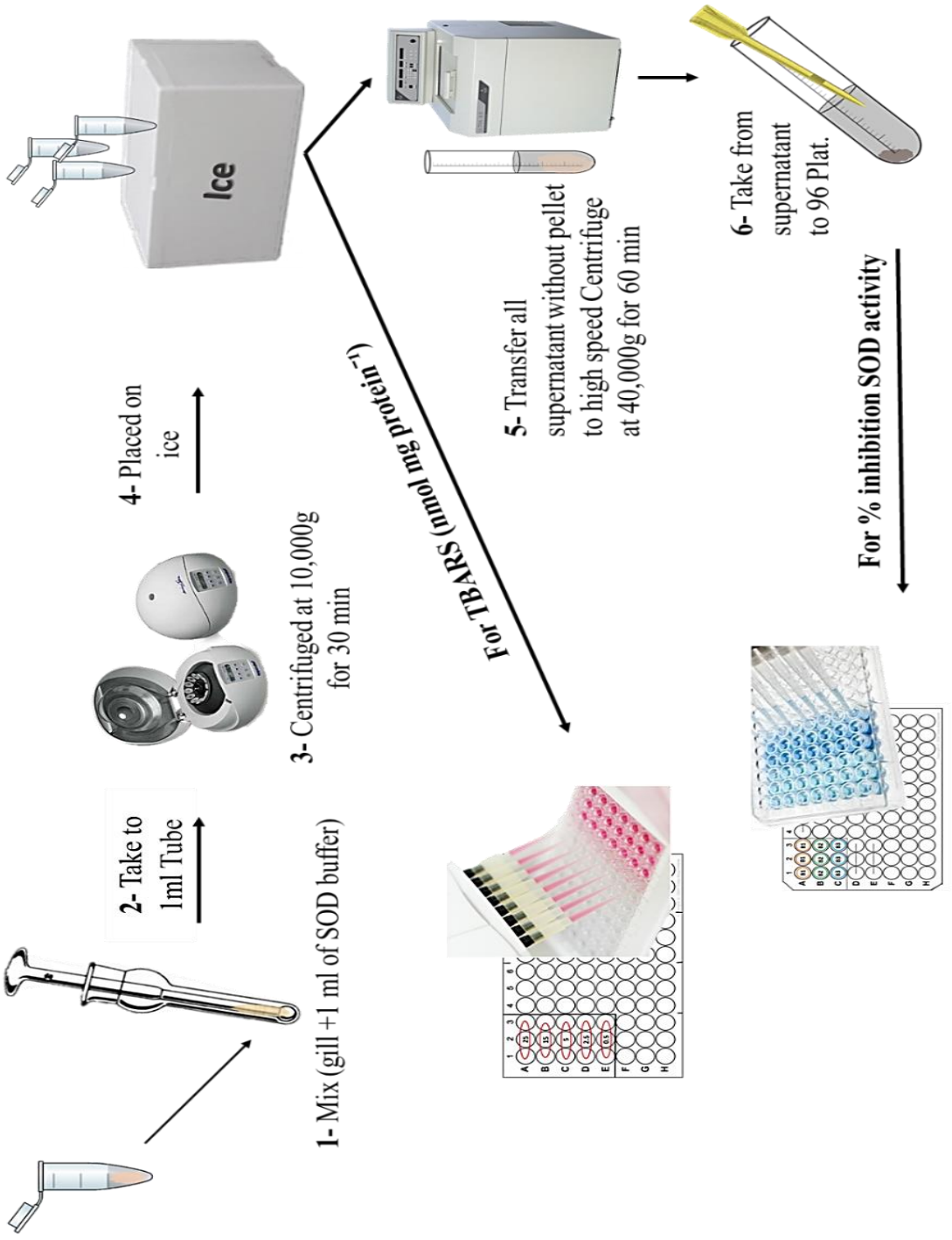


Figure 3.21: Summary procedure preparation tissue of SOD and TBARS assay

3.4.2.4.1 Superoxide Dismutase (SOD) Assay Kit -Working Solution (WST)

Superoxide dismutase (SOD) is an enzyme which catalyzes the partitioning of the superoxide to either an oxygen molecule (O_2^-) or hydrogen peroxide (H_2O_2), and this reduction can cause extensive cell damage if it is unregulated. To check for superoxide dismutase (SOD), a Sigma 19160 superoxide dismutase determination kit was used. Xanthine oxide (XO) is an enzyme which generates a reactive oxygen species. Its activity has a linear correlation to the rate of oxygen reduction, and hence it can be used to find the IC_{50} (that measures how much does need for a particular molecule/substance or SOD activity to inhibit some biological process by 50%), of processes which are affected by SOD activity. The SOD used here consisted of: 100µl of enzyme solution; 5ml working solution (WST); 100ml of buffer solution and 50ml of dilution buffer (Figure 3.22).

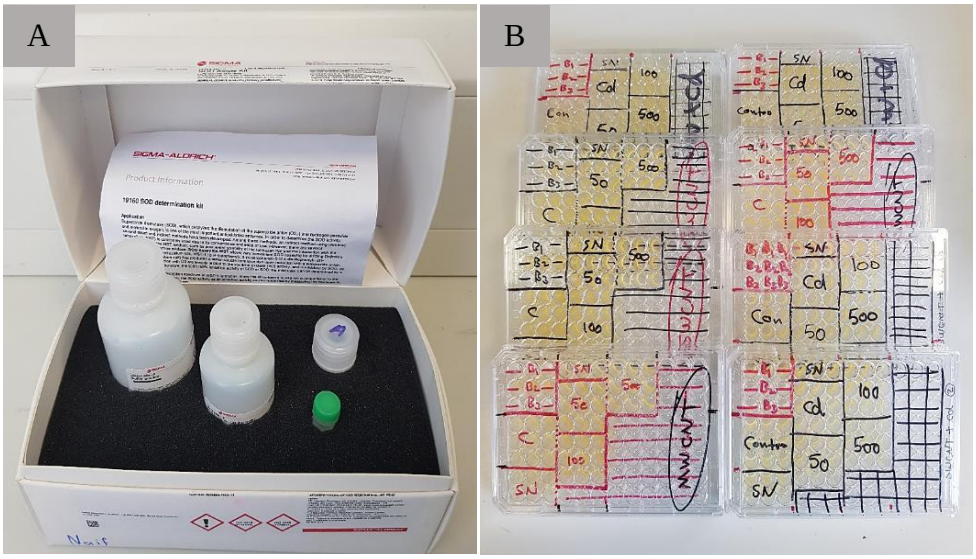


Figure 3.22: The SOD kit (WST solution, enzyme working solution, buffer solution and dilution buffer) (A), Prepared 96-well microplate, including samples and reagents (B).

Preparing a Working Solution

1- **Working solution**: 19ml of buffer solution was used to dilute 1ml of Water-Soluble Tetrazolium salt (WST).

2- **Enzyme solution:** the enzyme tube solution was placed in a centrifuge for five seconds to settle the enzymes down in the tube, and then 15µl of the solution was diluted down, using 2.5ml of the dilution buffer.

A ninety-six well microtitre plate was prepared, with 20µl of the cytosolic fraction, the supernatant, being drawn up and pipetted into each well, to which the reagents were added. Table 3.2 presents the amount of solution for each well, the SOD standard, each solution sample, and blanks 1, 2 and 3.

The plate was then placed in an incubator for twenty minutes at 50°C and readings were taken at 450nm, using a spectrophotometer (spectra Max M5) (Figure 3.23).

From this, SOD activity could be expressed as an inhibition percentage, and represented by the following equation:

Equation 3.1

$$\%\ \text{inhibition of SOD activity} = \frac{[(\text{Abs blank1} - \text{Abs blank3}) - (\text{Abs sample x} - \text{Abs blank2})]}{(\text{Abs blank1} - \text{Abs blank3})} \times 100$$

Table 3.2: Amount of solution for each well and blanks 1, 2 and 3

Solution	Sample	Blank 1	Blank 2	Blank 3
Sample solution	20 µl	----------	20 µl any sample	----------
ddH2O	----------	20 µl	----------	20 µl
WST	200 µl	200 µl	200 µl	200 µl
Enzyme working solution	20 µl	20 µl	----------	----------
Dilution buffer	----------	----------	20 µl	20 µl

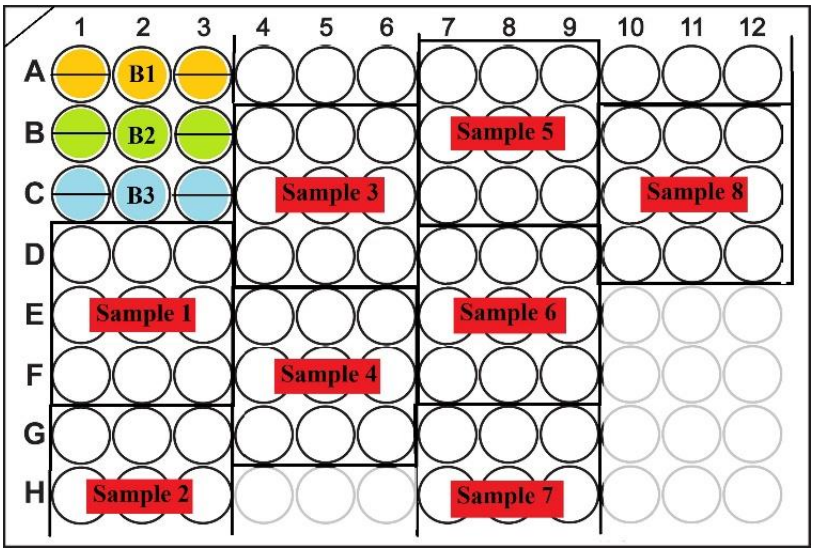


Figure 3.23: A prepared 96-well microplate layout for SOD Assay

3.4.2.4.2 Thiobarbituric Acid Reactive Substances

Determination of thiobarbituric acid reactive substances (TBARS) was carried out by emulating a previous method used for trout, as reported by Smith *et al*. (2007). Using a ninety-six well microtitre plate (Figure 3.24) as before, 75 µL of thiobarbituric acid (TBA); 40µL of gill homogenate; 140 µL of phosphate-buffered saline (PBS); 10µL of butylated hydroxytoluene (BHT) and 50µL of trichloroacetic acid (TCA) was added to each well (Figure 3.25). The microtitre plate was then incubated at 50°C for sixty minutes, and, using a spectrophotometer (spectra Max M5), readings at 530 nm and 630 nm were taken (correction for turbidity 530–630 nm) and compared against a standard series of tetraethoxypropane (TEP), at 0.5 nmol, 2.5 nmol, 5 nmol, 15 nmol, and 25 nmol, which can be expressed as nMol mg protein^{-1}. Using an equation formulated by Bradford (1976) (Figure 3.26). TBARS concentration and total homogenate protein could then be calculated:

Equation 3.2

$$Abs = A_{530nm} - A_{630nm}$$

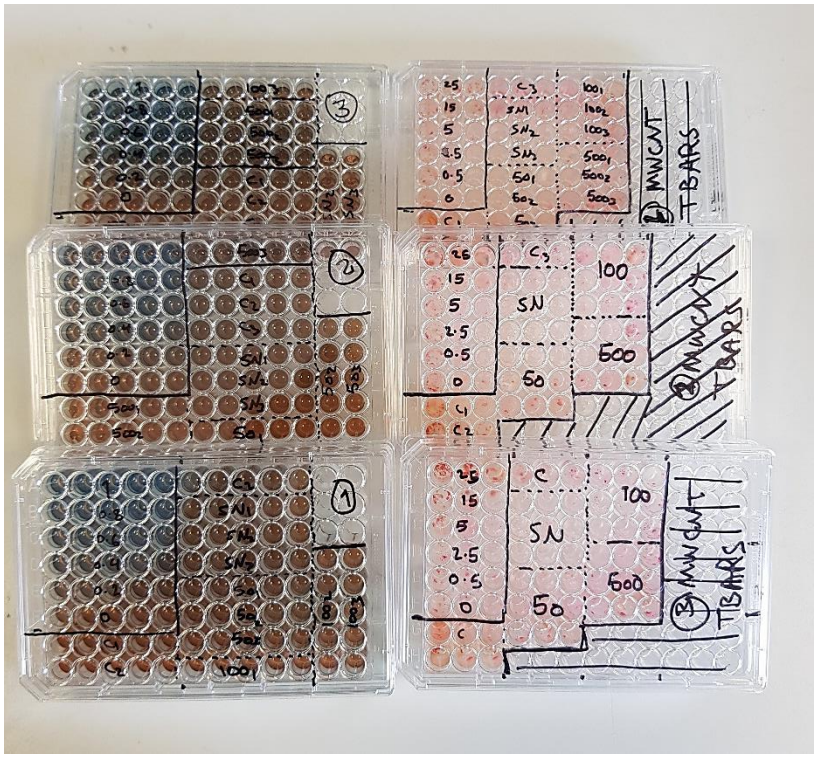


Figure 3.24: Prepared TBARS and BSA plates after incubation period of 60 minutes (n=3).

Reagents

- 1 mol/L butylated hydroxytoluene (2,6-Di-O-tert-butyl-4-methylphenol) (BHT)
- PBS 1 mmol/L EDTA (pH 7.4)
- 50% (w/v) trichloroacetic acid (TCA) in distilled water
- 1.3% (w/v) thiobarbituric acid (TBA) in 0.3% (w/v) NaOH
- 0.5–25 nmol 1,1,3,3-tetraethoxypropane (TEP in ethanol) (1 mol=220.31 g L^{-1}).

Method

Using a glass homogenizer, the tissue was homogenized, by hand and on ice, in five volumes of PBS .

Added in triplicate to each well in a 96-well microtitre plate:

40 µl of homogenate

10µl BHT

140µl PBS

50µl TCA

75µl TBA

TBARS standard were prepared as described below (Table 3.3)

Stock TEP: 1 mol=220.31 g L^{-1}

1nMol = 220.31 ng L^{-1}

25nMol ml^{-1} = 5507µg

1µL=220.31ug

Table 3.3: TBARS standard preparation calculation

Step	TEP (µl)		Ethanol (µl)	Conc (µMol)
0	0	+	2000	0
1	100 (of 1M stock TEP)		9900	10,000
2	10 of 1		3900	25
3	600 of 2		336	15
4	200 of 3		400	5
5	400 of 4		400	2.5
6	150 of 5		600	0.5

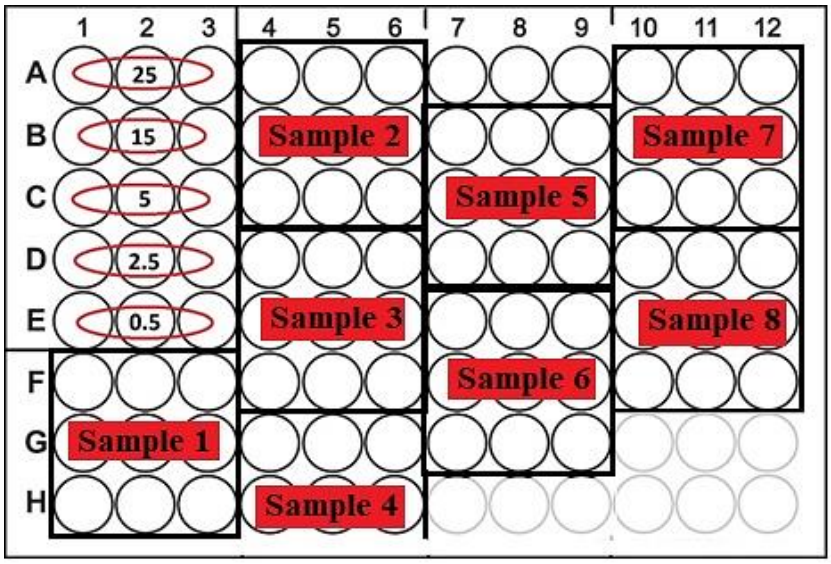


Figure 3.25: 96-well microplate layouts for TBARS Assay

Added in triplicate to each well in a 96-well microtitre plate (Figure 3.25):

40µl 0.5-25 nmol TEP

10µl BHT

140µl PBS

50µl TCA

75µl TBA

Cooled on ice and incubated at 60°C for one hour.

Absorbance was recorded twice, firstly at 530nm and secondly at 630nm.

Determine total protein concentration in homogenate (Bradford, 1976) (Figure 3.26).

Prepare a bovine serum albumin (BSA) standard stock solution (1 mg ml^{-1})

Prepare a dilution series (1, 0.8, 0.6, 0.4, 0.2, 0 mg ml^{-1})

Pipette 10 µl of each standard dilution to five separate wells.

Pipette 290 µl of Bradford reagent.

Incubate for 5 mins, read absorption at 595nm.

Pipette 10 µl of each homogenate into 5 replicate wells

Homogenates will probably need diluting 1:10

Pipette 290 µl of Bradford reagent.

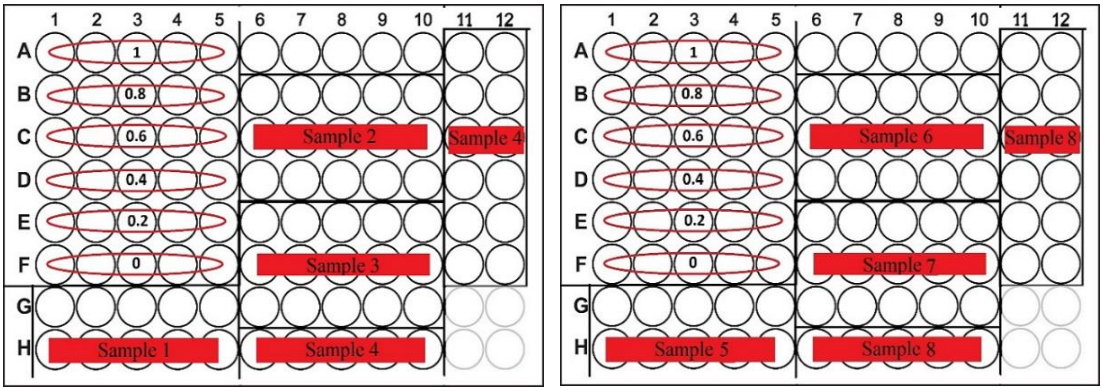


Figure 3.26: 96-well microplate layouts for TBARS Assay (BSA plate)

Incubate for 5 mins, read absorption at 595nm

Equation 3.3

$$TBARS = \frac{Abs}{Protein}\ nmol\ mg\ protein^{-1}$$

3.5 The Effect of CNTs Bioavailability on Sediment Associated Contamination

This part of the thesis was designed to ascertain two things: the interaction of CNTs bioavailability with metals in the marine environment, and the potential toxicity of CNTs with sediments associated-contamination.

Bioavailability is a measure of how efficiently a substance, in this case dissolved metals cadmium (Cd) and zinc (Zn) separately and in combination with either SWCNTs or MWCNTs, is absorbed by the target organism (Griscom and Fisher, (2004), in this case the cockle, *C. edule*. This was carried out in three stages: initially with CNTs or dissolved Cd or Zn on their own, and then with the CNTs combined with either Cd or Zn, and then combined with both Cd and Zn. This section examines the processes used in the collection of the cockles, how they were prepared, and the relevant parts extracted and analysed. It specifies which chemical concentrations were chosen and why, and how they were prepared. It then describes and explains the biomarker tests: cell viability, comet assay, SOD, TBARS and also the chemical analysis of the biomarkers and toxicity endpoints, the buffers that were used and the assay protocols that were applied.

3.5.1 Choosing and Preparing Genotoxic Chemicals

Cd is known as a genotoxic agent; as a result it was chosen for this experiment. It is used in the compound Cd chloride in an aqueous solution, from Sigma-Aldrich (Ref:

catalogue C5081). Zn is an essential, diamagnetic mineral from the Group 12 stable of metals in the periodic table and also has known toxic potential. It has many uses, from a propellant used in model rockets to dietary supplementation, it is essential for DNA repair and Zn deficiencies can cause depression and diarrhea (Pruski and Dixon, 2002; Prasad, 2003). Here, it is used in the form of Zn sulphate from BDH (Ref: catalogue 306215J) in an aqueous solution.

3.5.2 Dynamic light scattering (DLS) and Zeta potential

In this study, to ascertain the surface charges of different SWCNTs and MWCNTs and their interactions with dissolved metals (Cd^{+2} and Zn^{+2}), a zeta potential was measured at a pH of 8.4. Concentrations of each SWCNT and MWCNT at 50µg L^{-1}, 100µg L^{-1}, and 500µg L^{-1} and concentration of either **Cd** 0.001µM or **Zn** 1.0µM were placed in suspension, with seawater. These suspensions were left for two hours in ultrasonic dispersion to dismantle any agglomeration obtained during the preparation of the solution. A measurement was then taken with a red laser (Malvern Nano-ZS Zetasizer, Reference. No 2011143) with a wavelength of 633nm, using DLS and zeta potential measurements.

3.5.3 Interaction of the CNTs and sediment-associated contaminants

As has been shown experimentally, CNTs have the ability to absorb many different contaminants (Jackson *et al.*, 2013; Madani *et al.*, 2013). Consequently, they may also affect the toxicity of environmental pollutants, including the toxic metals Cd and Zn.

To better understand the relationship between SWCNTs, MWCNTs and metals (in this case dissolved Cd and Zn), a chemical analysis was carried out. As shown in Figure 3.27, sea water was placed in three beakers and spiked with both single-wall and multi-walled carbon nanotubes, followed by three different mixtures of metals: Cd only; Zn only, and a mixture of the two. Using a MSE Mistral 1000 centrifuge, the SWCNT and MWCNT samples were spun for ten minutes at 3000rpm (2520 RCF), and then the metals were analyzed separately, first in the pellet, and then in the supernatant. Both were examined by flame Atomic Absorbance Spectrometer to confirm whether the metals were present or absent in the pellet and supernatant sample.

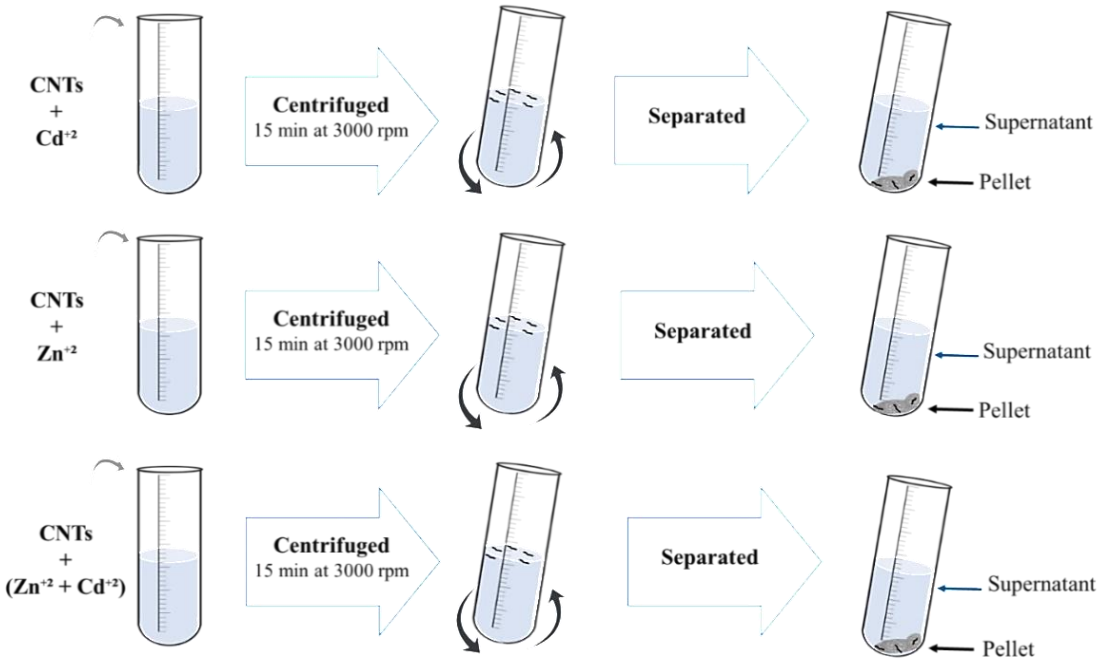


Figure 3.27: The determination of metal (Cd^{+2}, Zn^{+2}) partitioning behaviour in an aqueous suspension of SWCNTs and MWCNTs.

3.5.4 The Interaction of the CNTs and sediment-associated contaminants within the Cockles

This part of thesis evaluates if the CNTs affected the uptake of the toxic metals Cd and Zn into the cockles. This analysis was performed using AAS.

3.5.4.1 Treatment

12 filtered, aerated seawater (33±1 ppt; 14°C) beakers (2L) were filled with 0.5 kg of washed type 2 sediments, which their metal concentration was measured separately as control sediments (sample 1), and 1 litre of seawater was carefully added and any re-

suspended sediments left to settle. Air pumps were used to maintain a constant environment. The water in each tank was then spiked in triplicate to three replicates of:

Sample 1. Control Sediments
Sample 2. Control Sample cockles

Sample 3. SWCNTs 100µg L^{-1}.
Sample 4. MWCNTs 100µg L^{-1}.

Sample 5. Cd 0.001µM.
Sample 6. Zn 1.0 µM.

Sample 7. SWCNTs 100µg L^{-1} + Cd 0.001µM
Sample 8. SWCNTs 100µg L^{-1} + Zn 1.0µM
Sample 9. SWCNTs 100µg L^{-1} + Cd 0.001µM + Zn 1.0µM

Sample 10. MWCNTs 100µg L^{-1} + Cd 0.001µM
Sample 11. MWCNTs 100µg L^{-1} + Zn 1.0µM
Sample 12. MWCNTs 100µg L^{-1} + Cd 0.001µM + Zn 1.0µM

Three cockles, all between 3-4cms long, were gently introduced to each beaker. The first beaker was used as a control. The concentrations were chosen as effective concentrations, defined through preliminary experiments and were in line with previous work with mussels (Al-Shaeri *et al*., 2013). All the spiking described was carried out *in vivo* for 72 hours, without changing the water during that period to concentrate the concentrations. This experiment was repeated three times.

3.5.4.2 Using AAS to Determine Spiked Metals (Cd, Zn) in Cockle Gills

Protocol described by Liu and Kueh (2005)

After exposure for seventy-two hours, the cockles were removed and two gills from each side of the bivalve were extracted using tweezers, and frozen at -80ºC until ready for examination. To get an accurate measurement, first the gills had to be prepared and weighed. The frozen gills were thawed out, and then, still wet, added to 10ml glass beakers, which had been weighed first (Figure 3.28). The beakers were placed in an oven for a minimum of twenty-fours at 60°C to dry out the gills, then the beaker and gills were weighed again to find the dry weight of the gills (Table 3.4).

Table 3.4: Empty beaker weight and dry weight of cockles' gills

Chemical exposure	Empty beaker wt.	Dry wt. gill	Net dry wt.
Control Samples	50.871g	51.701g	0.83g
SWCNTs 100µg L^{-1} Samples	50.433g	51.423g	0.99g
MWCNTs 100µg L^{-1} Samples	50.520g	51.47g	0.95g
Cd 0.001µM Samples	50.294g	51.114g	0.82g
Zn 1.0µM Samples	50.602g	51.522g	0.92g
SWCNTs 100µg L^{-1} + Cd Samples	49.980g	50.71g	0.73g
MWCNTs 100µg L^{-1} + Cd Samples	50.127g	50.898g	0.771g
SWCNTs 100µg L^{-1} + Zn Samples	49.615g	50.275g	0.66g
MWCNTs 100µg L^{-1} + Zn Samples	50.106g	50.799g	0.693g
SWCNTs 100µg L^{-1} +Cd+Zn Samples	49.810g	50.47g	0.66g
MWCNTs 100µgL^{-1}+Cd+Zn Samples	45.878g	46.638g	0.76g

To be able to extract the metals from the gill tissue, nitric acid (NHO_3) at a 70% v/v concentration was added to each sample and this solution was left for twenty-four hours. After this time, the nitric acid mixture was diluted with 5ml of distilled water, and then left in a water bath (SUB36, Reference: 2008254, from Grant) for twenty-four hours at 60°C. The samples were then filtered, and distilled water was poured into each beaker up to 50ml. As before, AAS was used to analyse the gill samples (Figure 3.27).

Figure 3.28: Dried cockle gill tissue and filtered extracts for AAS

3.5.5 The Toxicity of CNTs with sediment-associated contaminants

The goal of this experiment was to investigate how different environments affect the toxicity of cockles after exposure to SWCNTs and MWCNTs together with sediment-associated contaminants such as (Cd and Zn), in three different exposure treatments, to identify the differences between the SWCNTs' and MWCNTs' behaviour in seawater and investigate the bioavailability of CNTs to sediment associated contamination and sediment-dwelling cockles and, lastly, to examine the cockles feeding behaviour and uptake of the CNTs from the environment. It will also confirm whether the toxicity of the CNTs becomes greater in seawater or in the surface of sediment or when the CNTs are mixed into the sediment.

3.5.5.1 Exposure Conditions (Treatments)

The aim of this part of the study was to determine the impact of three treatment scenarios on the toxicity of MWCNT and SWCNT suspensions with heavy metals. In the first treatments, SWCNTs and MWCNTs plus Cd, Zn, or a combination of the two, were spiked into the water column; in the second treatment, SWCNTs and MWCNTs plus Cd, Zn, or a combination of the two, were spiked onto the surface of sediment, and in the third they were mixed with the sediment. Three different concentrations were used to determinate the concentrations-response relationship. Separate negative control tanks were run in parallel. All the spiking described was carried out in vivo for 72 hours, without changing the water during that period to concentrate the concentrations. The results of all the assays mentioned above were used to explain

feeding mechanisms and the uptake of SWCNTs and MWCNTs with or without metals, and the sediment-associated contaminants level of toxicity, in presence of CNTs, if they are taken up by cockles.

3.5.5.1.1 Treatment 1 (Water Spiked) with Metals (Cd and Zn)

The aim of this treatment was to assess whether the cockles had absorbed the CNTs and the dissolved metals directly from the seawater, or from sediments, to try to confirm whether the spread of CNTs would be more toxic and whether the CNTs would have more effect on the organisms or on the sediment itself.

Glass tanks of 13×5L size were filled with 500g of washed sediment, and 1L of seawater was carefully added and any re-suspended sediment left to settle. Three healthy cockles were introduced into the system by placing them onto the sediment surface where they were allowed to bury themselves. Then, in a 1 mL tube, the CNTs were mixed with dissolved metals (Cd and Zn, separately and combined) using different concentrations for each group, as listed below. Then the water in each tank was spiked with the three different groups of SRNOM-dispersed CNTs with metals (Cd and Zn), as listed below and left to settle onto the surface of the sediment and cockles.

The cockles were exposed in triplicate to:

Group 1.

Control

Cd 0.001μM
Zn 1.0 μM
Cd 0.001μM + Zn 1.0 μM

SWCNTs 50μg L^{-1}
SWCNTs 50μg L^{-1} + Cd 0.001μM
SWCNTs 50μg L^{-1} + Zn 1.0μM
SWCNTs 50μg L^{-1} + Cd 0.001μM + Zn 1.0μM

MWCNTs 50μg L^{-1}
MWCNTs 50μg L^{-1} + Cd 0.001μM
MWCNTs 50μg L^{-1} + Zn 1.0μM
MWCNTs 50μg L^{-1} + Cd 0.001μM + Zn 1.0μM

Group 2.

Control

Cd 0.001μM
Zn 1.0 μM
Cd 0.001μM + Zn 1.0μM

SWCNTs 100μg L^{-1}
SWCNTs 100μg L^{-1} + Cd 0.001μM
SWCNTs 100μg L^{-1} + Zn 1.0μM
SWCNTs 100μg L^{-1} + Cd 0.001μM + Zn 1.0μM

MWCNTs 100μg L^{-1}
MWCNTs 100μg L^{-1} + Cd 0.001μM
MWCNTs 100μg L^{-1} + Zn 1.0μM
MWCNTs 100μg L^{-1}+ Cd 0.001μM + Zn 1.0μM

Group 3.

Control

Cd 0.001µM
Zn 1.0 µM
Cd 0.001µM + Zn 1.0 µM

SWCNTs 500µg L^{-1}
SWCNTs 500µg L^{-1} + Cd 0.001µM
SWCNTs 500µg L^{-1} + Zn 1.0µM
SWCNTs 500µg L^{-1} + Cd 0.001µM + Zn 1.0µM
MWCNTs 500µg L^{-1}
MWCNTs 500µg L^{-1} + Cd 0.001µM
MWCNTs 500µg L^{-1} + Zn 1.0µM
MWCNTs 500µg L^{-1} + Cd 0.001µM + Zn 1.0µM

3.5.4.1.2 Treatment Two (Surface Spiked) with Metals (Cd and Zn)

The aim of this treatment was to assess whether CNTs and dissolved metals would pose a threat to the organisms if exposed via the water, compared to the sediment surface.

Glass tanks of size 13×5L glass were filled with 500g of washed sediment and 1L of seawater was carefully added to each and any re-suspended sediment left to settle. Then, in a 1 mL tube, the CNTs were mixed with dissolved metals (Cd and Zn separately and combined) using different concentrations for each group, as listed below. The water in each tank was then spiked with the three different groups of SRNOM-dispersed CNTs with metals (Cd and Zn), as listed below, and left to settle onto the surface of the sediment. Healthy cockles were introduced into the system by placing them onto the sediment surface and allowing them to bury themselves.

The cockles were exposed in triplicate to:

Group 1.

Control

Cd 0.001μM
Zn 1.0 μM
Cd 0.001μM + Zn 1.0 μM

SWCNTs 50μg L^{-1}
SWCNTs 50μg L^{-1} + Cd 0.001μM
SWCNTs 50μg L^{-1} + Zn 1.0μM
SWCNTs 50μg L^{-1} + Cd 0.001μM + Zn 1.0μM

MWCNTs 50μg L^{-1}
MWCNTs 50μg L^{-1} + Cd 0.001μM
MWCNTs 50μg L^{-1} + Zn 1.0μM
MWCNTs 50μg L^{-1} + Cd 0.001μM + Zn 1.0μM

Group 2.

Control

Cd 0.001μM
Zn 1.0 μM
Cd 0.001μM + Zn 1.0 μM

SWCNTs 100μg L^{-1}
SWCNTs 100μg L^{-1} + Cd 0.001μM
SWCNTs 100μg L^{-1} + Zn 1.0μM
SWCNTs 100μg L^{-1} + Cd 0.001μM + Zn 1.0μM

MWCNTs 100μg L^{-1}
MWCNTs 100μg L^{-1} + Cd 0.001μM
MWCNTs 100μg L^{-1} + Zn 1.0μM
MWCNTs 100μg L^{-1} + Cd 0.001μM + Zn 1.0μM

Group 3.

Control

Cd 0.001µM
Zn 1.0 µM
Cd 0.001µM + Zn 1.0 µM

SWCNTs 500µg L^{-1}
SWCNTs 500µg L^{-1} + Cd 0.001µM
SWCNTs 500µg L^{-1} + Zn 1.0µM
SWCNTs 500µg L^{-1} + Cd 0.001µM + Zn 1.0µM

MWCNTs 500µg L^{-1}
MWCNTs 500µg L^{-1} + Cd 0.001µM
MWCNTs 500µg L^{-1} + Zn 1.0µM
MWCNTs 500µg L^{-1} + Cd 0.001µM + Zn 1.0µM

3.5.4.1.3 Treatment Three Sediment Spiked with Metals (Cd and Zn)

The aim of this treatment was to assess at what level of uptake and availability sediment dwelling organisms, such as cockles will be affected, and to confirm whether the level of absorption of CNTs into a cockle's cells (and therefore potential toxicity) is dependent on the feeding environment (water, surface or sediment).

Glass tanks of 13×5L size were prepared as described above. However, in this experiment, the SRNOM-dispersed CNTs were mixed with sediment-associated contaminants (Cd and Zn separately and combined) and the sediment in a Thermo-MAXQ 3000 shaker for five minutes at 200 rpm before adding seawater. This gave the following nominal sediment concentrations (which were concentrations of CNTs equivalent to treatment 1: 0.1 µg.g^{-1}, 0.2 µg. $g^{-1}$and 1 µg. g^{-1}) for the three different

groups, as listed below. Seawater was added gently to avoid excessive resuspension of sediment, which was left to settle; cockles were then added as described above.

The cockles were exposed in triplicate to:

Group 1.

Control
Cd 0.001µM
Zn 1.0 µM
Cd 0.001µM + Zn 1.0 µM

SWCNTs 0.1 $\mu g.g^{-1}$
SWCNTs 0.1 $\mu g.g^{-1}$ + Cd 0.001µM
SWCNTs 0.1 $\mu g.g^{-1}$ + Zn 1.0µM
SWCNTs 0.1 $\mu g.g^{-1}$ + Cd 0.001µM + Zn 1.0µM

MWCNTs 0.1 $\mu g.g^{-1}$
MWCNTs 0.1 $\mu g.g^{-1}$ + Cd 0.001µM
MWCNTs 0.1 $\mu g.g^{-1}$ + Zn 1.0µM
MWCNTs 0.1 $\mu g.g^{-1}$ + Cd 0.001µM + Zn 1.0µM

Group 2.

Control

Cd 0.001µM
Zn 1.0 µM
Cd 0.001µM + Zn 1.0 µM

SWCNTs 0.2 $\mu g.g^{-1}$
SWCNTs 0.2 $\mu g.g^{-1}$ + Cd 0.001µM
SWCNTs 0.2 $\mu g.g^{-1}$ + Zn 1.0µM
SWCNTs 0.2 $\mu g.g^{-1}$ + Cd 0.001µM + Zn 1.0µM

MWCNTs 0.2 $\mu g.g^{-1}$
MWCNTs 0.2 $\mu g.g^{-1}$ + Cd 0.001µM
MWCNTs 0.2 $\mu g.g^{-1}$ + Zn 1.0µM
MWCNTs 0.2 $\mu g.g^{-1}$ + Cd 0.001µM + Zn 1.0µM

Group 3.

Control

Cd 0.001µM
Zn 1.0 µM
Cd 0.001µM + Zn 1.0 µM

SWCNTs 1 $\mu g.g^{-1}$
SWCNTs 1 $\mu g.g^{-1}$ + Cd 0.001µM
SWCNTs 1 $\mu g.g^{-1}$ + Zn 1.0µM
SWCNTs 1 $\mu g.g^{-1}$ + Cd 0.001µM + Zn 1.0µM

MWCNTs 1 $\mu g.g^{-1}$
MWCNTs 1 $\mu g.g^{-1}$ + Cd 0.001µM
MWCNTs 1 $\mu g.g^{-1}$ + Zn 1.0µM
MWCNTs 1 $\mu g.g^{-1}$ + Cd 0.001µM + Zn 1.0µM

3.5.6 Biomarker analysis

The exact mechanisms which occur with the interaction of SWCNTs and MWCNTs, with sediment-associated contaminants (Cd and Zn), and how they may induce the toxic effects on cockles remain largely undiscovered. This part of the thesis looks at the metals, SWCNTs and MWCNTs, both in combination and in isolation, and if and how their potential genotoxicity will alter DNA, and how they affect cell viability using Trypan blue dye and any oxidative stress recorded, using SOD and TBARS respectively. These endpoints were established for all three treatments and contaminant combinations, as described above.

3.6 Data Analysis

Statistical analysis was carried out and the graphs were produced using GraphPad Prism Software. To enable correct interpretation of the data, where appropriate, an angular (or arcsine) transformation was used to give a linear graph. Arcsine transformation uses the function, calculated as two times the arcsine of the square root of the proportion, to give a result which can be more easily interpreted (Sokal and Rohlf, 2012). The DNA damage was expressed as the percentage of tail DNA (Lovell and Omori, 2008), and determined using the image analysis software package Comet Assay IV (Perceptive Instruments) and analysed with a one-way analysis of variance (ANOVA), after arcsine transformation, followed by a Tukey all-pairwise multiple comparison procedure (Sparks, 2000). Each of the comet data points was made up of three replicates of 150 individual measurements, 50 from each of the three animals in each replicate tank. Therefore, it is considered that $n = 3$ was appropriate to pick up fluctuations in background DNA damage. The results after transformation of the SOD activity data were plotted on an ANOVA (one-way analysis of variance) graph, then a Tukey all-pairwise multiple comparison procedure was used to determine the statistical differences between the treatment groups and the control when checking the normality and the equal variance assumption tests (Sparks, 2000). Statistical significance was accepted at $P<0.05$. The results after transformation were plotted on an ANOVA (one-way analysis of variance) graph and the data statistically analysed, as described above. The thiobarbituric acid reactive substances data did not require

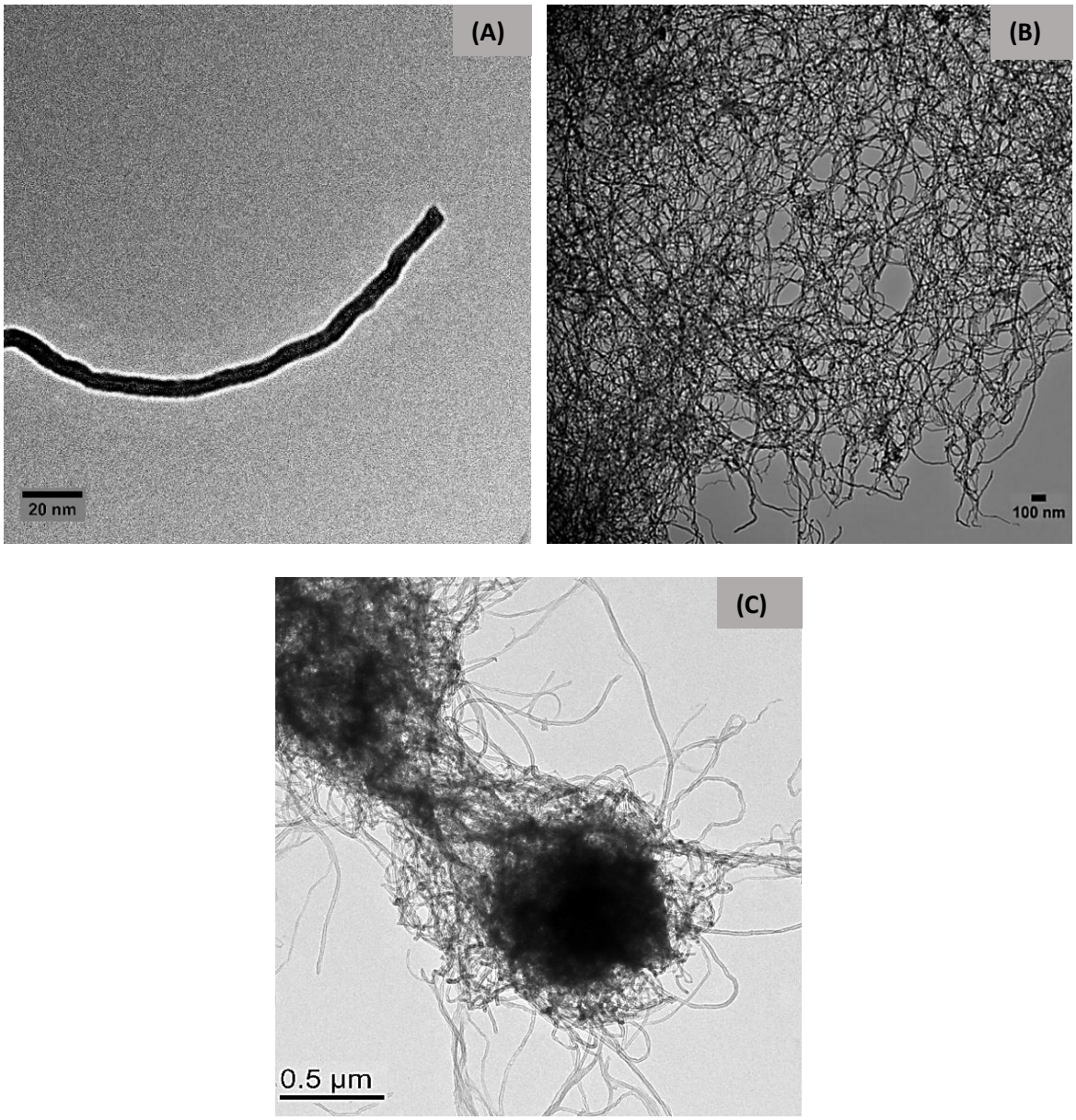
transformation and were subjected directly to an ANOVA/Tukey analysis, as described above.

CHAPTER 4 RESULTS

4.1 Characterisation of Stock SWCNTs and MWCNTs

4.1.1 Transmission Electronic Microscope (TEM)

The characterisation of the CNTs (MWCNTs and SWCNTs) was carried out in the stock suspension and under exposure conditions. Visual examination of CNT dispersion using TEM was only possible in the stock preparations. The dispersion of CNT agglomerates in 0.02% SRNOM for the stock preparation (1 g L^{-1}) with and without sonication is shown in Figure 4.1. Following the dispersion process, smaller agglomerates were found in the SWCNT dispersion than in the MWCNT dispersion. The SWCNTs appeared as small tubes with a single wall, resembling a single layer of rolled-up wire (Figure 4.1 A-C). The MWCNTs also appeared as small tubes, but with two or more concentric layers with various diameters and lengths; they were also more agglomerated at the same concentration (Figure 4.1 D-F). The TEM micrographs indicated high purity nanotube samples, with amorphous carbon rarely noted (Figure 4.1). Nanotube lengths ranged from hundreds of nanometres to a few micrometres. The diameters of MWCNTs ranged from 6 to 9 nm, whereas for SWCNTs they were typically ≈ 1 nm.


(A)
20 nm
(B)
100 nm
(C)
0.5 µm

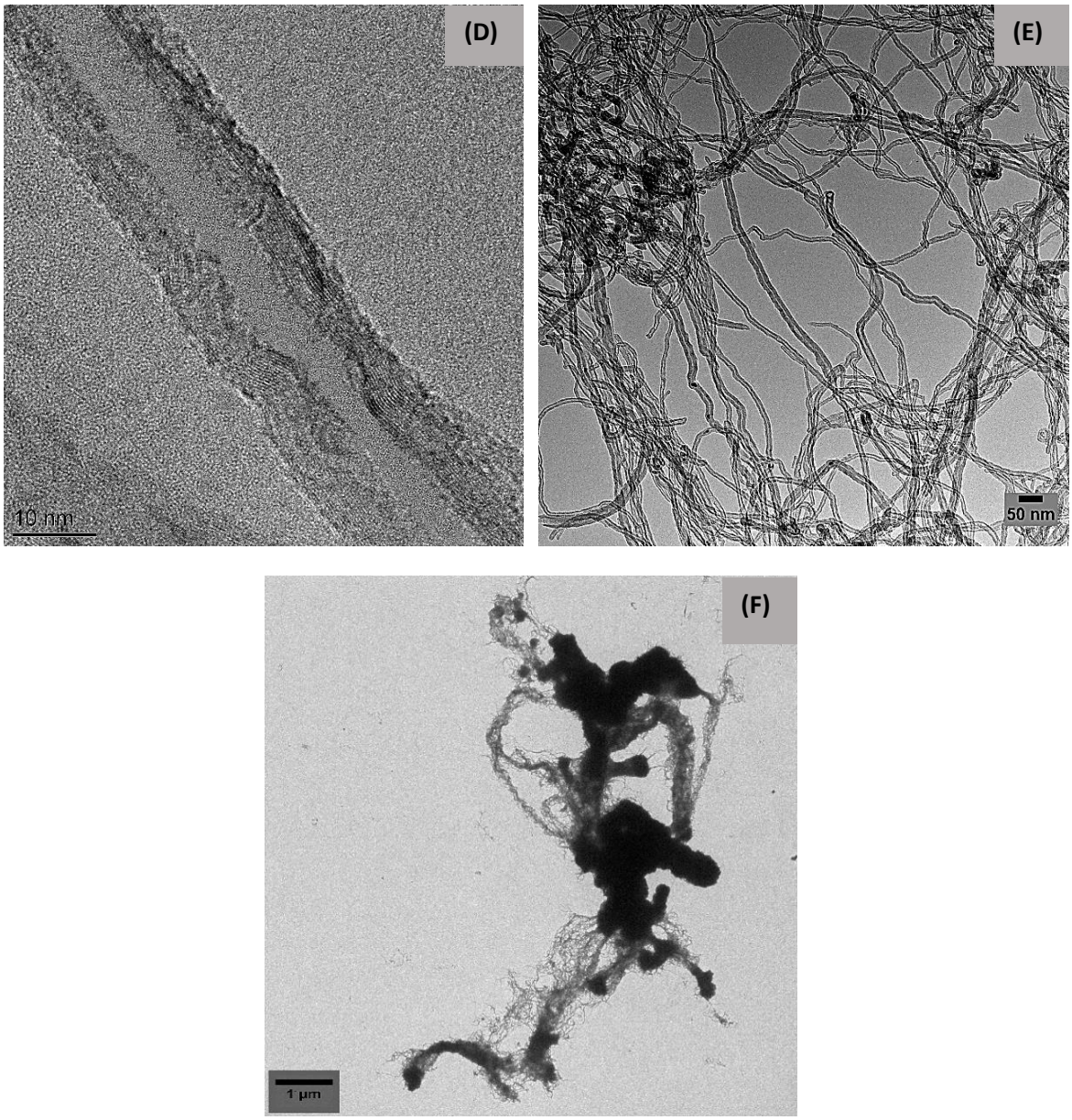


Figure 4.1: Transmission Electron Micrographs of CNT dispersion preparations (1 mg L^1 in 0.02% Suwannee River natural organic matter): single-walled carbon nanotube (SWCNT) stock with sonication (A-B) and without (C); multi-walled carbon nanotube (MWCNT) stock with sonication (D-E) and without (F).

4.1.2 Dynamic light scattering (DLS) and Zeta potential

The agglomerates for both types of CNTs under exposure conditions were negatively charged: the SWCNTs' charge ranged between -8 and -15 mV, while that of the MWCNTs ranged between -11 and -19 mV (Table 4.1). The approximate sizes for both types were found to increase in a concentration-dependent manner (Table 4.1). The SWCNTs' agglomerate length was 1264.66 nm while that of the MWCNTs was 2305 nm at the same concentration of 50 µg L^{-1}. The zeta potential of MWCNTs indicated a higher agglomerate stability compared to SWCNTs.

Table 4.1: Average aggregate size and zeta potential of SWCNT and MWCNT particulates at different concentrations suspended in a water medium and seawater (under exposure conditions), measured by DLS[1].

CNT Types	CNT (µg L^{-1})	DLS (nm)	Zeta potential of CNTs in water	Zeta potential of CNTs in (seawater)
SWCNTs	50	1264.66	-3.22	-8.46
	100	4150.33	-5.35	-13.02
	500	6022.45	-8.64	-15.14
MWCNTs	50	2505.32	-6.31	-11.46
	100	5900.7	-8.34	-15.62
	500	7331.63	-9.54	-19.12

[1] DLS = dynamic light scattering;
pH 8.4, salinity 32 (±1) ‰.

4.1.3 Raman spectroscopy

A typical Raman spectrum acquired from CNT stock is shown in Figure 4.2, which clearly shows the characteristic peaks of SWCNTs (G band) at 1,584 cm^{-1}. At low frequency, radial breathing mode (RBM) was at 268 cm^{-1}. Similarly, Figure 4.3 shows the characteristic peaks of MWCNTs (D band) at 1,330 cm^{1}, and at low frequency, (G' band) at 2,646 cm^{-1}. Both sets of spectra were collected using a ×50, 0.75 numerical aperture microscope objective lens (Leica N-plan). Acquisition time was 1 s, laser wavelength was 785 nm and power was 5 mW.

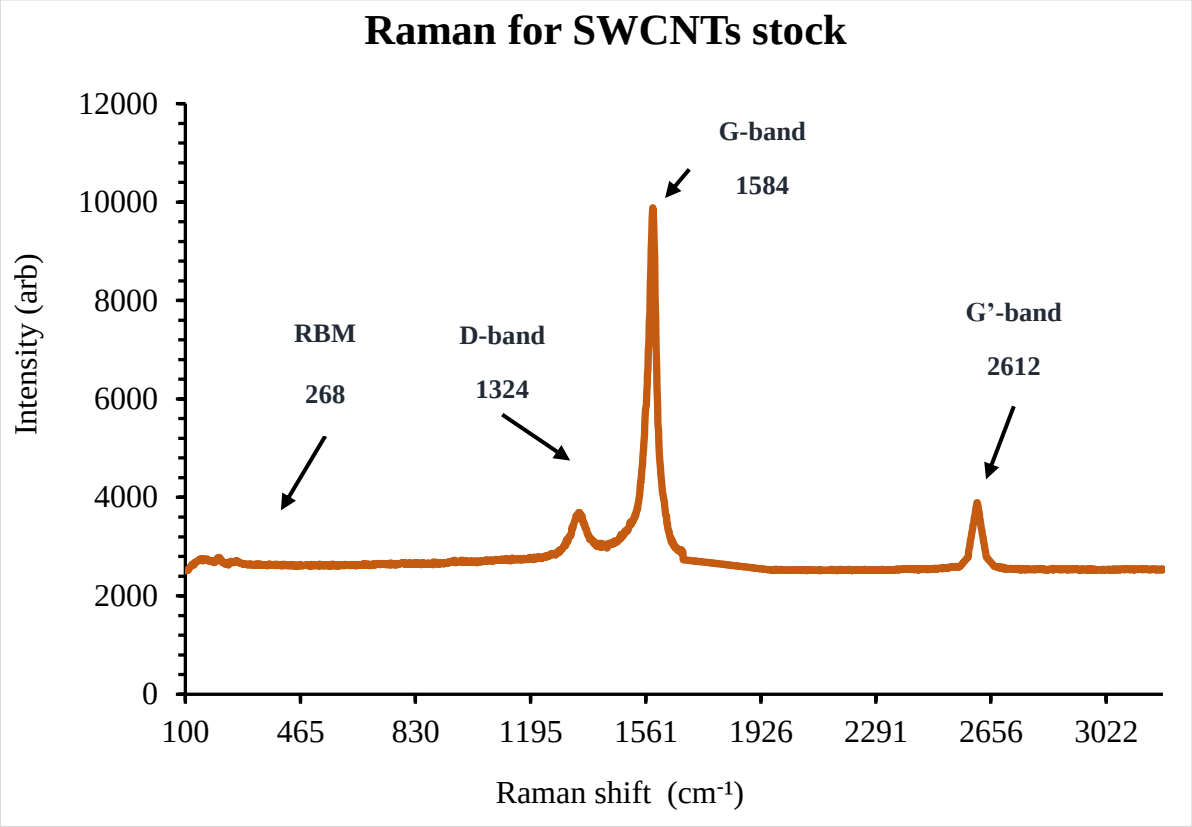


Figure 4.2: Spectrum from SWCNT stock clearly showing the characteristic peaks of SWCNTs: radial breathing mode (RBM) at 268 cm^{-1}, D band at 1,324 cm^{-1}, G band at 1,584 cm^{-1} and G' band at 2,612 cm^{-1}.

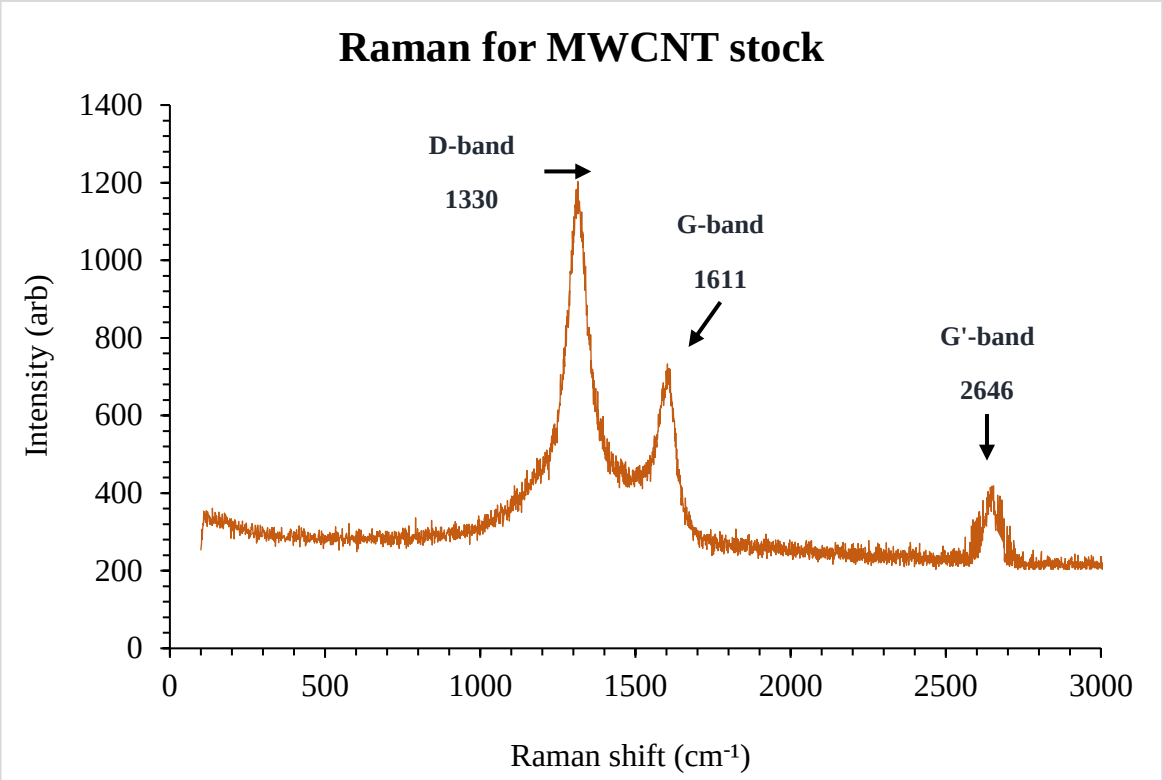


Figure 4.3: Spectrum from MWCNT stock, clearly showing the characteristic peaks of MWCNTs: D band at 1,330 cm^{-1}, G band at 1,611 cm^{-1} and G' band at 2,646 cm^{-1}.

4.2 The Bioavailability of SWCNTs and MWCNTs to Sediment-dwelling Cockles

4.2.1 Sediments

Sediment granulometry was determined using a stacked sieve shaker. The artificial sediment granulometry results and organic matter content are presented in Table 4.2. The sediment was composed of particles sized between 1000 µm and 63 µm, with the majority of particles found to be in the range of 355–250 µm. Based on sediment classification, it is clearly shown that the type of sediment is sand (medium sand) (Wentworth, 1922). The organic matter content was very low, as would be expected from washed sand.

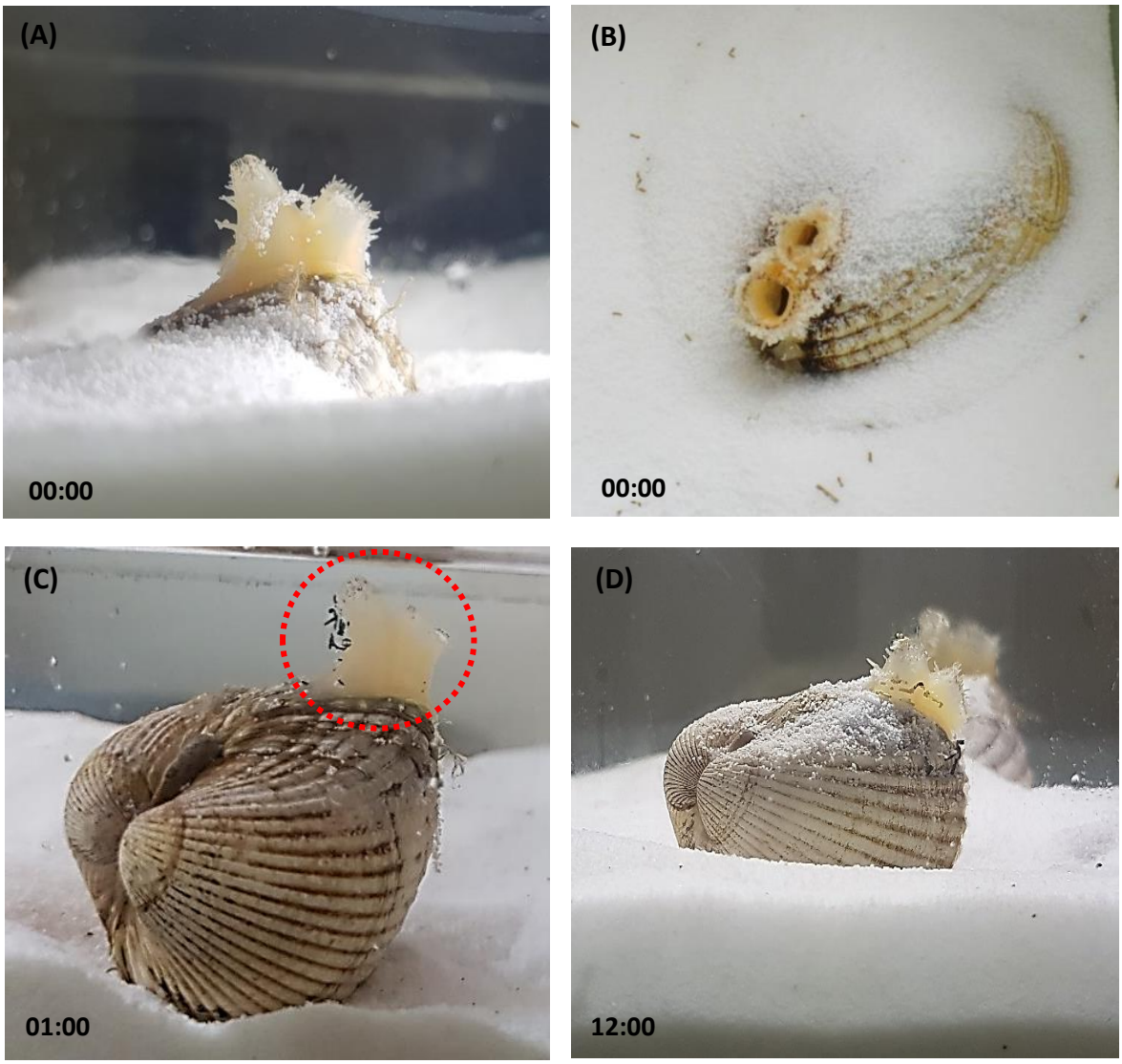
Table 4.2: Characterisation of Exposed Sediments

Grain size	%	Total Organic Matter in sediment
1000–355 µm	6.1	0.07%
355–250 µm	76.5	
250–125 µm	16.4	
125–63 µm	1.0	

4.2.2 CNTs bioavailability and interaction

4.2.2.1 CNT-environment interaction

Figure 4.4 shows the cockle in the laboratory, buried in sediment and exposed to the CNTs for 72 hours. In the early stages, the cockle remains buried under the sediment while the siphon is above the sediment to feed and filter the required nutrients, as shown in Figure 4.4A & B. After one hour, the cockle stays on the surface of the sediment while the inhalant siphon remains open for food uptake, as shown in Figure 4.4C. The figure clearly shows the agglomeration of CNTs on the inhalant siphon, which is absorbing them from the surface of the sediment and water column. After 12 hours of exposure, Figure 4.4D shows clearly the pseudofaecal matter released by the cockles. After 24 hours of exposure, the cockle releases the faecal material from the exhalant siphon into the habitat, after digesting and filtering the required nutrients, as shown in Figure 4.4E. After 36 hours of exposure, the faeces are shown on the surface of the sediment, separated from the agglomerated or aggregated CNTs, as shown in Figure 4.4F. The cockle then buries itself under the sediment after 72 hours (Figure 4.4G), with the remains of some faecal matter and agglomerated or aggregated CNTs are visible on the surface of the sediment.


(A)
00:00
(B)
00:00
(C)
01:00
(D)
12:00

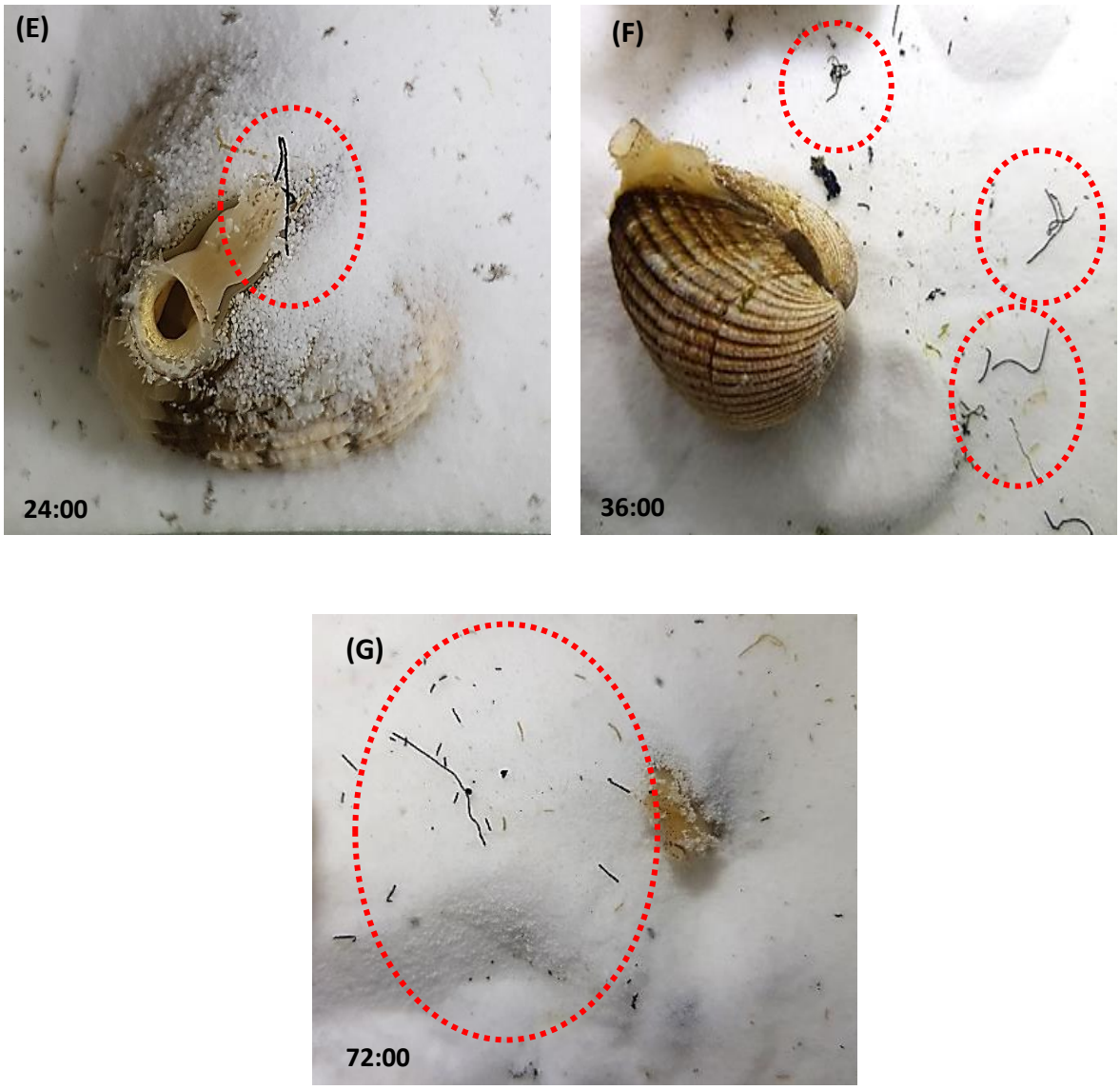


Figure 4.4: Cockle's interaction with the environment. A-B: cockle starts to expel CNTs. C: agglomerate black nanotubes still attached to the inhalant siphon of the cockle. D: cockle emits the pseudofaecal material. E: cockle emits faecal material. F-G: cockle's faecal matter on the sediment's surface.

4.2.2.2 CNT- Cockles interaction

After 72 hours of CNT exposure, the cockle was collected, and the valves gently opened using a small lab scalpel to avoid damaging the intestine. After this, the cockle was viewed under the dissecting microscope (stereo microscope) to examine the interaction of CNTs with the gill and digestive gland tissue. It could be seen that the CNT agglomerates were very effectively removed from the water column and sediment surface by the filter-feeding *C. edule*. The CNTs were coated in mucus (Figure 4.5A & B) on contact with the gills (Figure 4.5C & D), concentrated, transported across the gill surface and excreted as faeces (Figure 4.5E). It is unclear whether any CNTs were ingested. Some, however, remained attached to the gill membranes for at least 72 hours following exposure. The black residues retained within the gills were confirmed as CNTs using confocal Raman spectroscopy (Section 4.2.4).

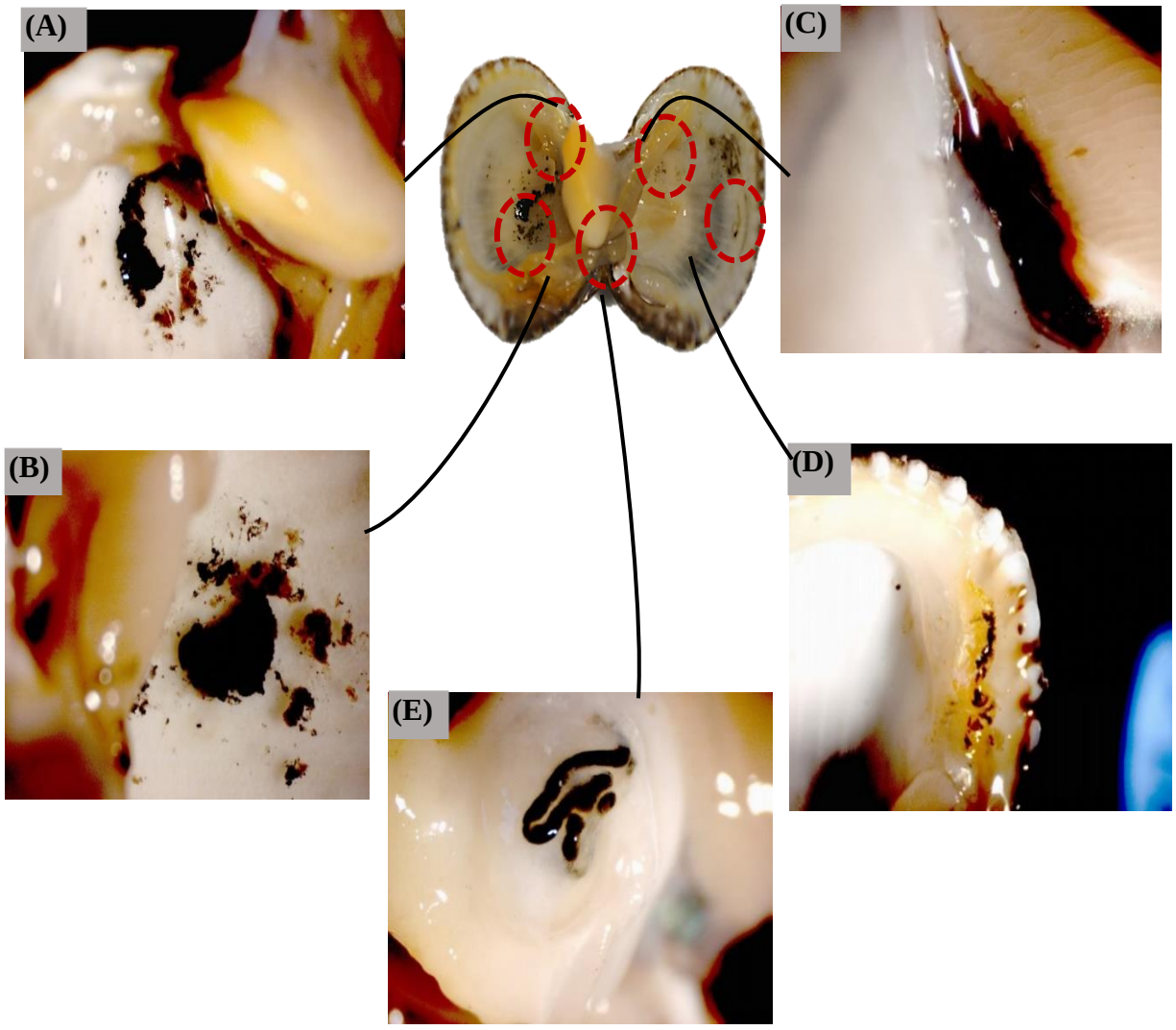
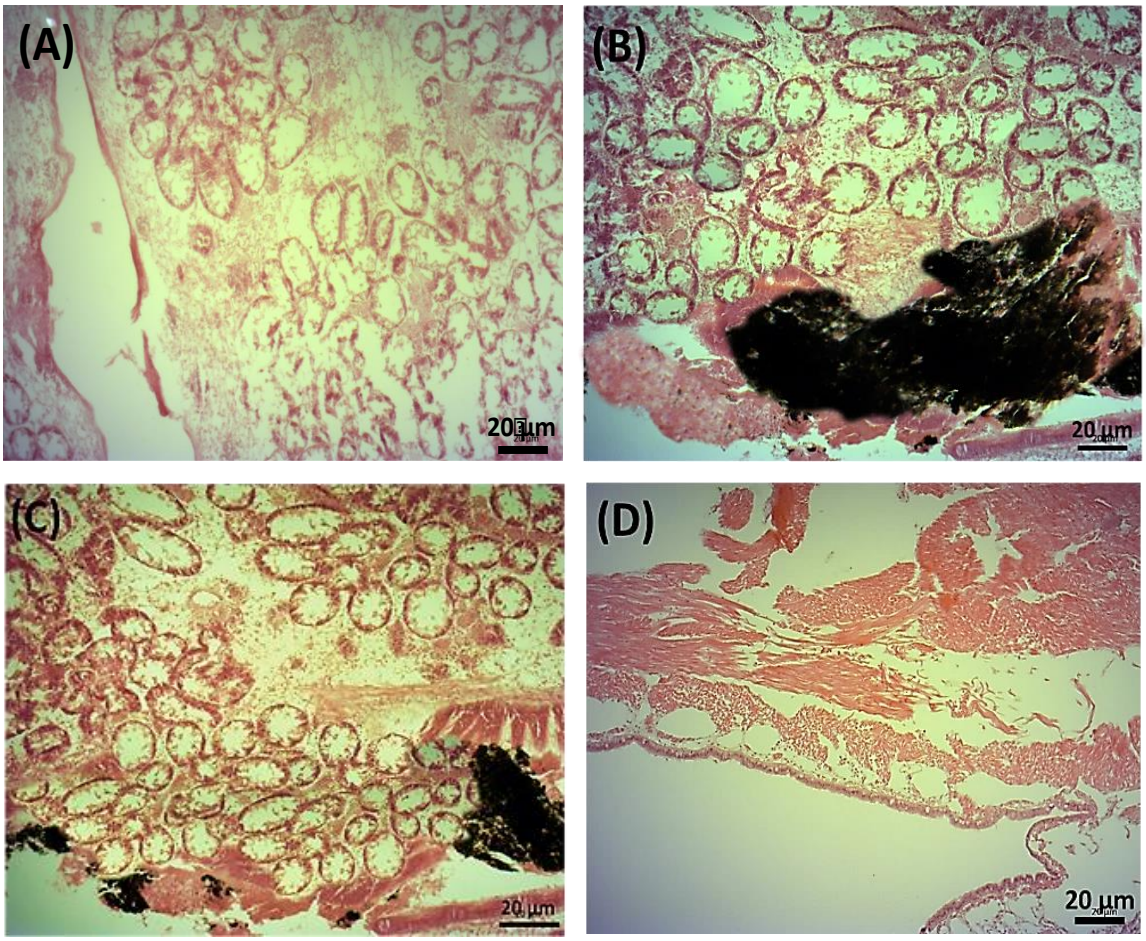


Figure 4.5: Dissection microscope observation was used as an initial method to observe the interaction of carbon nanotubes (CNTs) with the cockle's gills. The CNTs were coated in mucus (A-B). The CNTs on contact with the gills (C-D). Faeces inside the mucus (E).

4.2.3 Histological observation of transfer of CNTs from the environment to cockle tissues

Following exposure to CNT for 72 hours, histological sections of the cockle's digestive gland and gills were used to find evidence of any interaction between the cockle's tissue cells and CNTs (Figure 4.6A-F). Compared to the control (A), the agglomerated SWCNTs (B) and MWCNTs (C) were visible in the cockle's digestive gland. Moreover, comparing the control gill tissue (D), it can be observed that there are agglomerated SWCNTs (E) and MWCNTs (F) associated with the cockle's gill tissue.



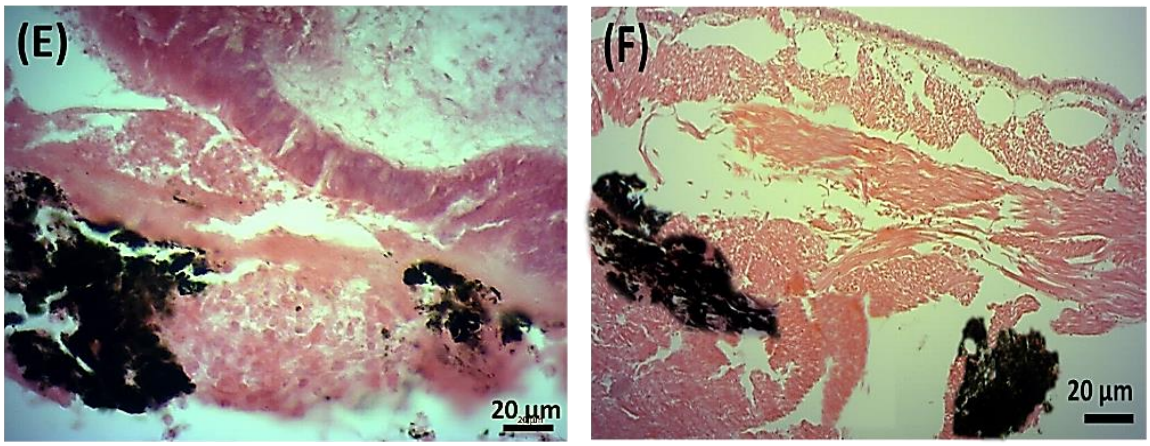
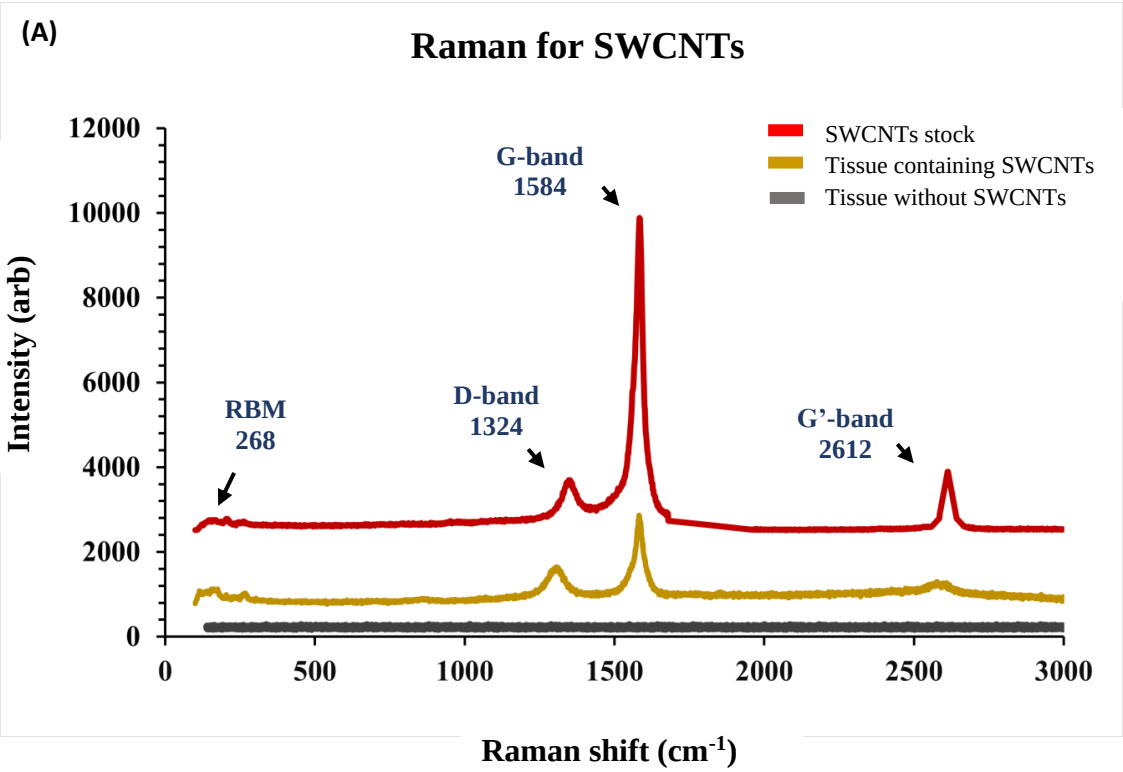


Figure 4.6: Histological sections. A: the control for the cockle's digestive gland tissue. B: sample of SWCNTs interacting with the cockle's digestive gland tissue. C: sample of MWCNTs interacting with the cockle's digestive gland tissue. D: control of the cockle's gill tissue. E: sample of SWCNTs interacting with the cockle's gill tissue. F: sample of MWCNTs interacting with the cockle's gill tissue.

4.2.4 Raman spectroscopy

4.2.4.1 Raman spectroscopy for cockle's gills

After exposing the cockle to both SWCNTs and MWCNTs for 72 hours, and after observing the cockle under the light microscope, Raman spectroscopy tests were performed on the agglomerated black materials on the cockle's gill to study their relationship with CNTs. The study was done to compare the spectra of the CNT stock, tissues containing CNTs and tissues without agglomerated CNTs, as shown in Figure 4.7. It can be seen that the peaks from the CNT stock and from the tissue containing CNTs show the same Raman shift in the G band, D band and G' band, but these are of less intensity for the tissues containing CNTs, due to the background of the tissue.



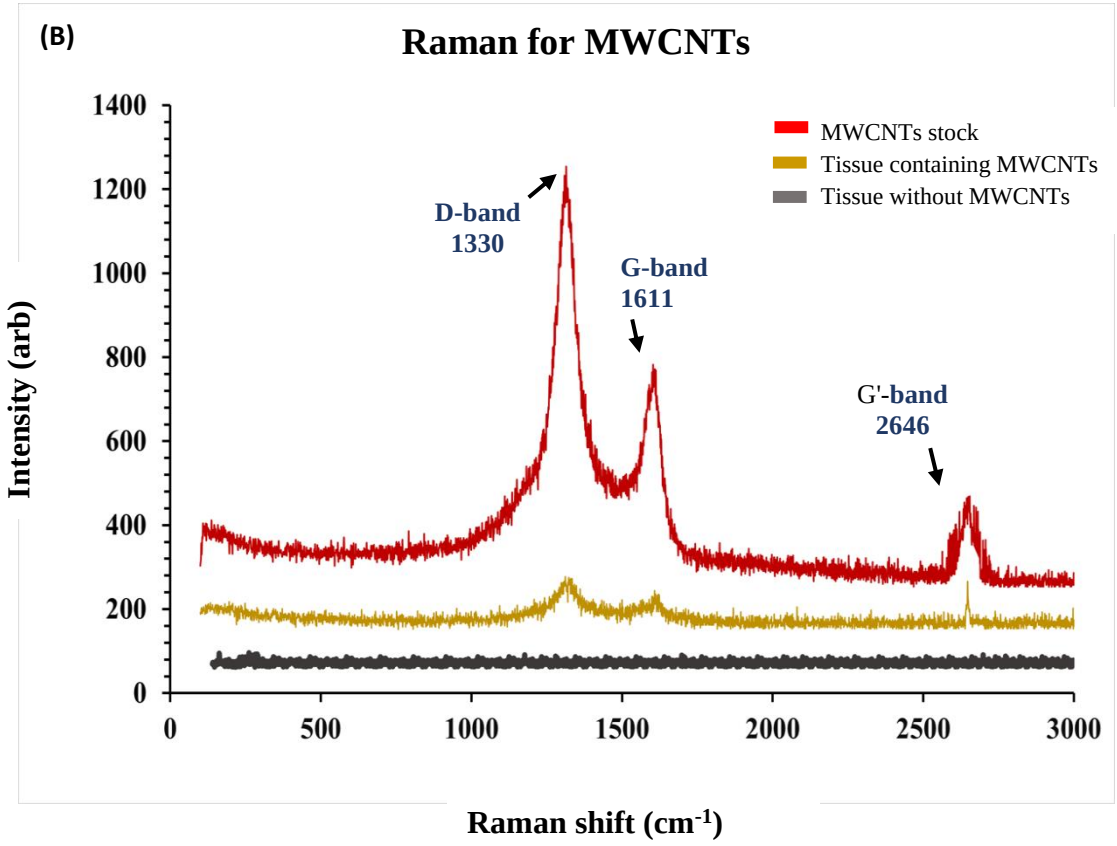


Figure 4.7: A: Representative Raman spectra acquired from SWCNT stock (top spectrum), cockle sample spiked with 100µg L^{-1} SWCNT for 72 hours (middle spectrum), and control gill tissue (bottom spectrum). B: Representative Raman spectra acquired from MWCNT stock (top spectrum), cockles sample spiked with 100µg L^{-1} MWCNT for 72 hrs (middle spectrum), and control gill tissue (bottom spectrum). Both sets of spectra were collected using a 50x, 0.75 numerical aperture microscope objective lens.

4.2.4.2 Raman spectroscopy for exposed sediment

After 72 hours of exposure, a sample was taken from the sediment and filtered, (as explained in Chapter 3). Subsequently, the filter (pore size = 0.45µm, Whatman) was tested under the Raman spectroscope as shown in Figure 4.8A and Figure 4.9A, to check any bioavailability of the CNTs in the sediment. The Raman mapping method was used to scan the chosen small site (25 µm × 25 µm) in the filter for 24 hours to detect any remaining CNTs (Figure 4.8B and Figure 4.9B). After detecting the CNTs' availability using the Raman mapping technique, the scattered light could then be observed as a signal acquired from the G band at 1,584 cm^{-1} for SWCNT (Figure 4.8C) and 1,611 cm^{-1} for MWCNT (Figure 4.9C). In order to confirm as many characteristic peaks (RBM, G band, G′ and D bands) of CNTs as possible, a wide range of Raman shifts (100–3500 cm^{-1}) was counted.

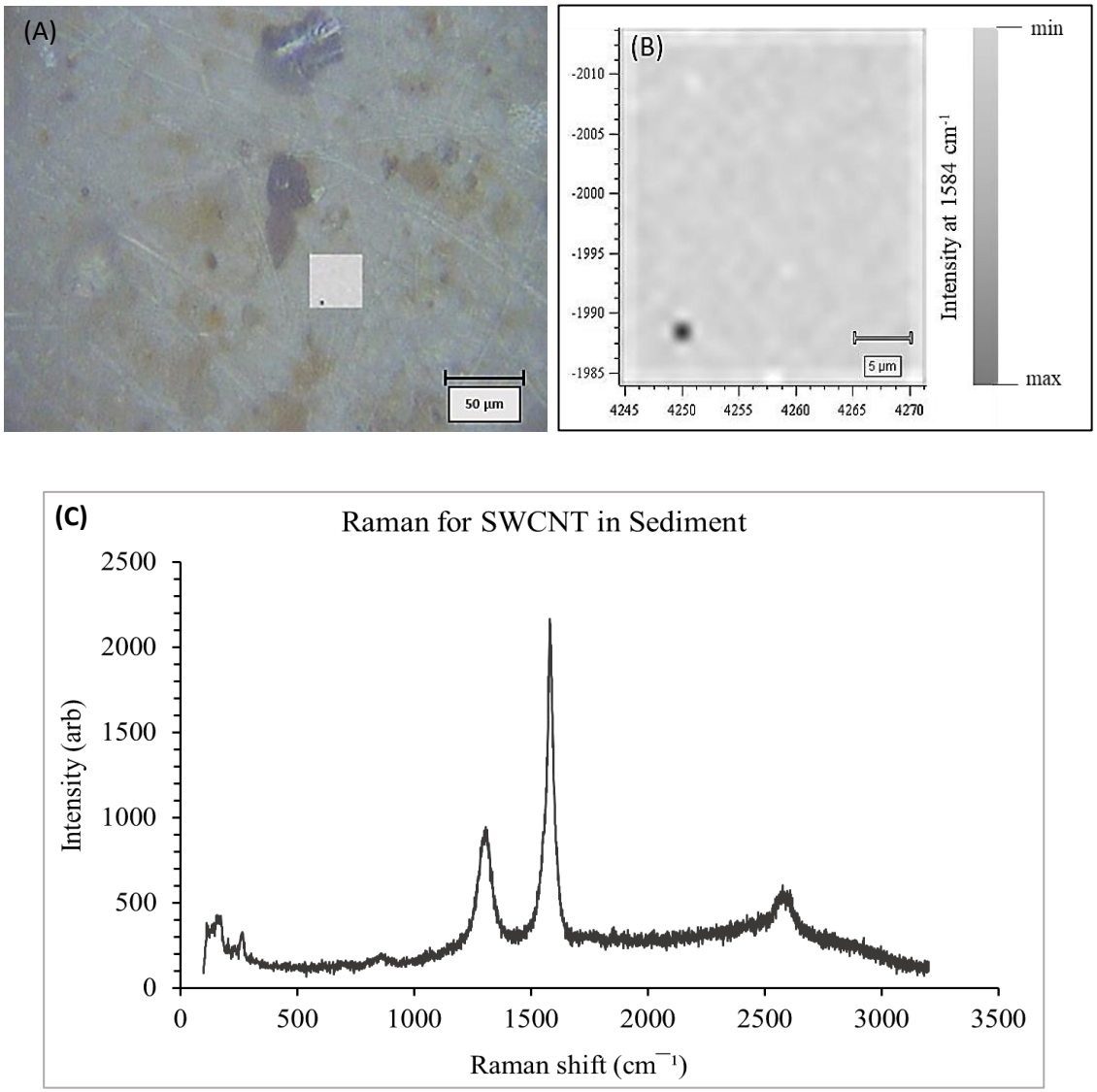


Figure 4.8: A: optical image (reflected light)/micrograph of partial SWCNTs suspended in sediment. The small square overlaid on the image is the intensity map of the signal acquired at the characteristic peaks of SWCNTs: G band at 1,584 cm^{-1} during the 2D scan. B: corresponding component Raman map (collected over 25 µm × 25 µm area at a sampling step of 1 µm in both x and y directions) over 21 hours scanning, showing heat map intensity. This indicates on a nominal scale the amount of SWCNTs present in that area, with black as maximum intensity and white as minimum. C: spectrum from SWCNTs, clearly showing the characteristic peaks of SWCNTs: RBM D band at 1,324 cm^{-1}, G band at 1,584 cm^{-1} and G' band at 2,612 cm^{-1}.

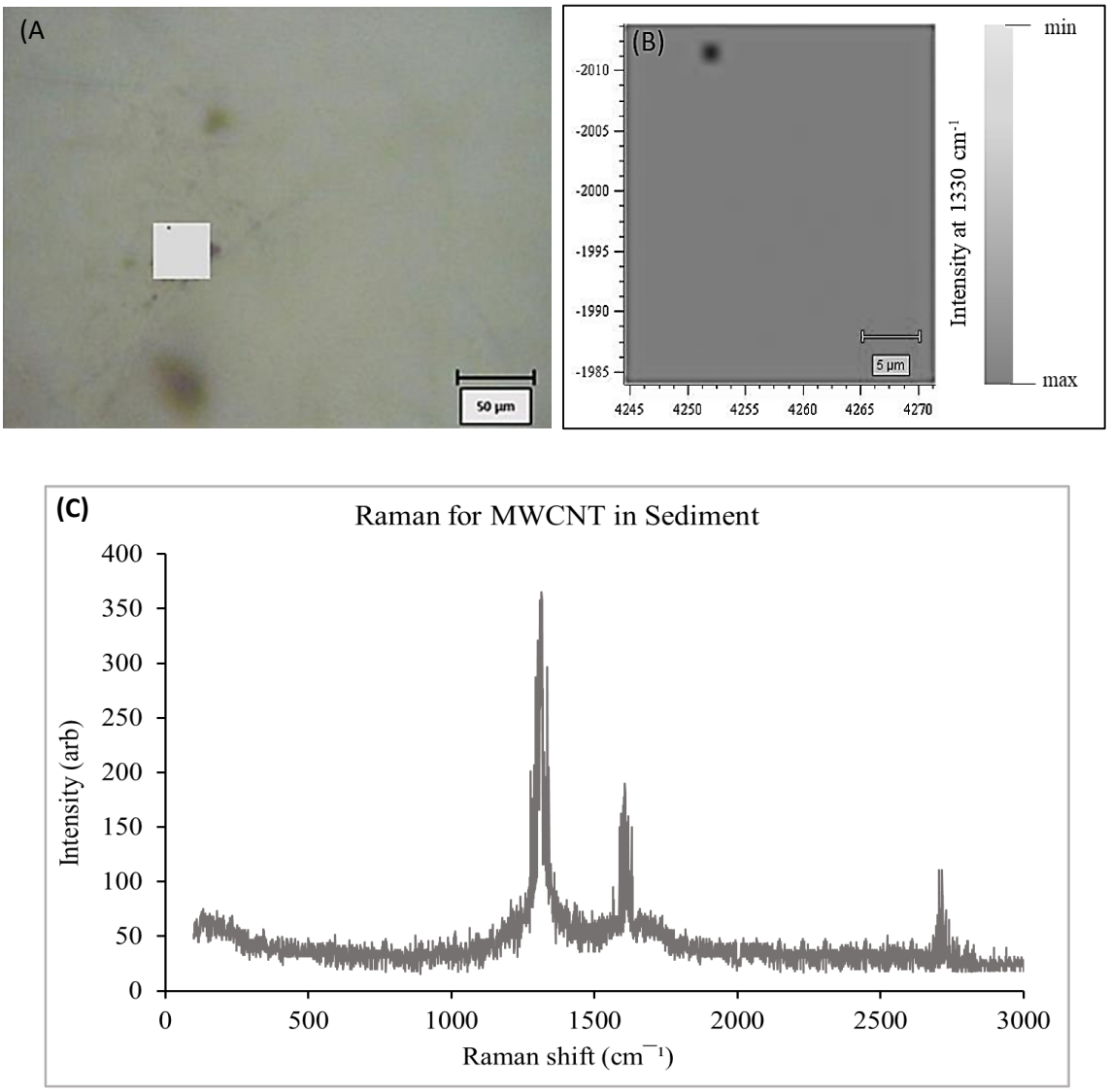
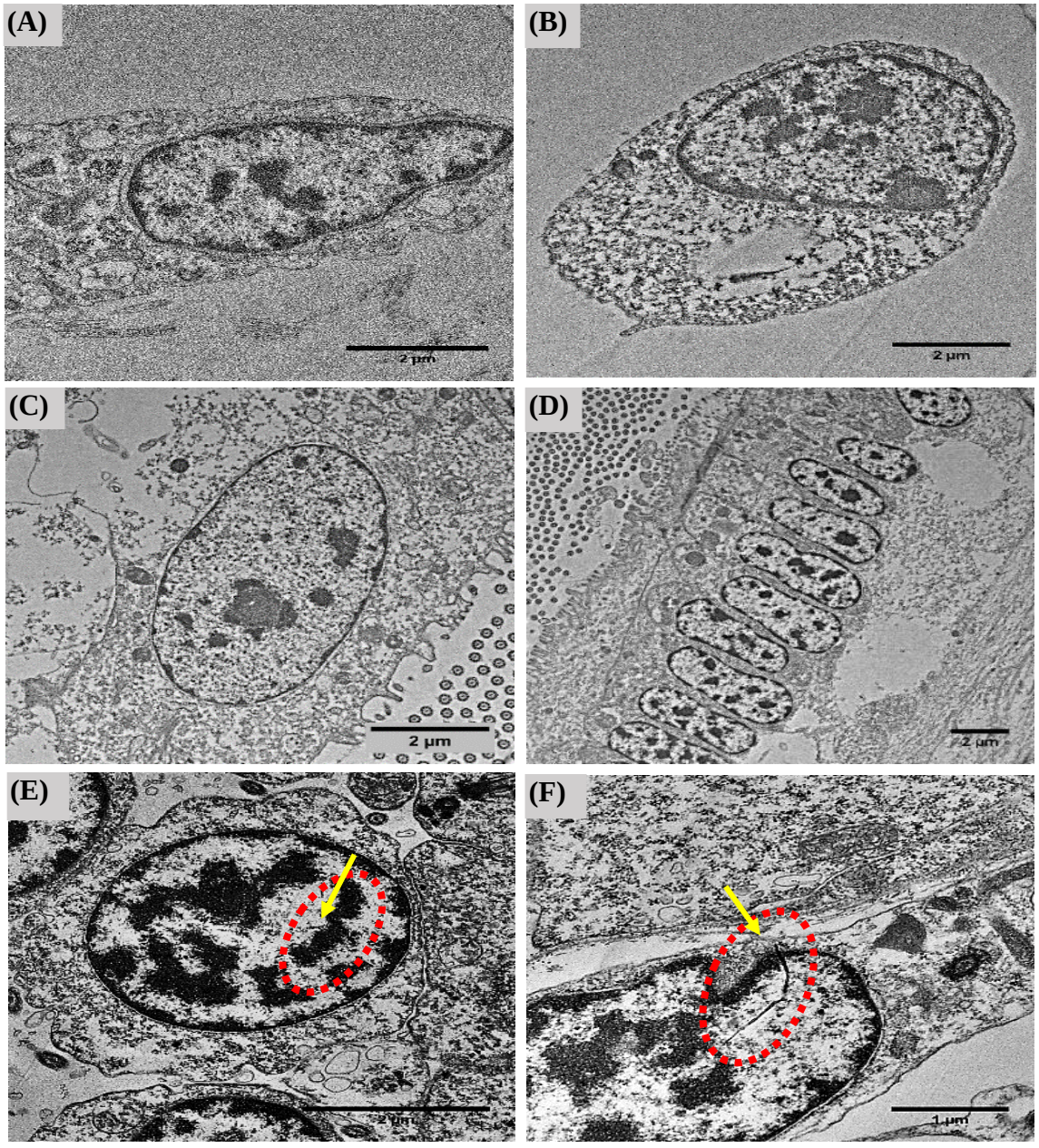


Figure 4.9: A: optical image (reflected light)/micrograph of partial MWCNTs suspended in sediment. The small square overlaid on the image is the intensity map of the signal acquired at the characteristic peaks of MWCNTs: D band at 1,330 cm^{-1} during the 2D scan. B: corresponding component Raman map (collected over 25 µm × 25 µm area at a sampling step of 1 µm in both x and y directions), showing heat map intensity. This indicates on a nominal scale the amount of MWCNTs present in that area, with black as maximum intensity and white as minimum. C: Spectrum from MWCNTs, clearly showing the characteristic peaks of MWCNTs: D band at 1,330 cm^{-1}, G band at 1,611 cm^{-1} and G' band at 2,646 cm^{-1}.

4.2.5 Transmission electronic microscope (TEM)

After 72 hours of exposure to the CNTs, some tissues from the cockle were tested under the TEM to examine whether the CNTs had been absorbed by the cells, had penetrated the cell membrane or remained outside the membrane, as shown in Figure 4.10.

Figures A and B show the healthy (control) cells of the digestive gland with no membrane breakage. There are no accumulations or breakages caused by CNTs. Figures C and D show the healthy (control) cells of the gills with no membrane breakage. There are no accumulations or breakages caused by CNTs. Figures E and F show the cells of the digestive gland that were exposed to SWCNTs. These have been taken up and have caused the cell membrane to break. Similarly, in the gill cells, a tube from the SWCNTs is clearly shown to have broken the cell membrane. On the other hand, Figures G and H show the digestive gland cells of the cockles exposed to MWCNTs. In this case, the MWCNTs did not cause membrane breakage in the digestive gland cells and they stayed on the edge of the membrane without breaking it (Figure G) or they stayed outside the gill cells (Figure H).


(A)
2 µm
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(C)
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(D)
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(E)
(F)
1 µm

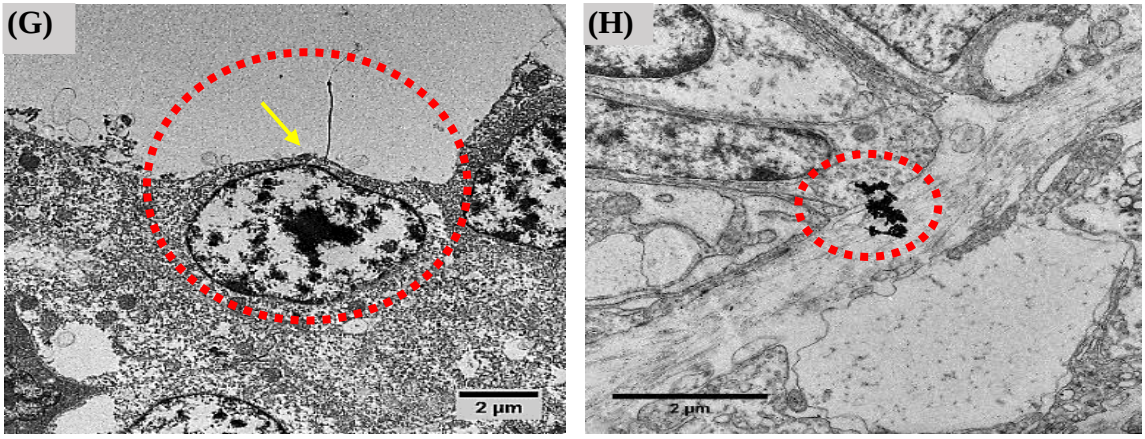


Figure 4.10: TEM images of control digestive gland cells (A and B) and control gill cells (C and D) of the cockle. The interaction of the cockle's digestive gland cells (E) and gill cells (F) with SWCNTs. The interaction the cockle's digestive gland cells (G) and gill cells (H) with MWCNTs. Cell membrane breakage is indicated by yellow arrows and internalisation of the CNTs is circled in red.

4.3 The Toxicity of SWCNTs and MWCNTs to Sediment-dwelling Cockles

4.3.1 Cell viability

The Trypan blue approach only expresses the percentage of live and dead cells under a light microscope, where dead cells appear in blue by taking up the blue dye. The cell viability results obtained from the Trypan blue technique showed that the cell viability decreased in a concentration-dependent manner when exposed to both forms of CNTs, seperatly, at nominal concentrations of 50 μgL^{-1}, 100 μgL^{-1} and 500 μgL^{-1} in treatments 1 and 2, and the equivalent concentrations of 0.1 $\mu g.g^{-1}$, 0.2 $\mu g.g^{-1}$ and 1 $\mu g.g^{-1}$ in treatment 3 for 72 hours (Table 4.3).

The cell viability results showed that there was significant effect on cell viability in the haemocytes of *C. edule* only when exposed at $\geq$100 μgL^{-1} in treatments 1 and 2, and ≥ 0.2 $\mu g.g^{-1}$ in treatment 3. It was observed that there was little difference between the percentage of live cells in the presence of SWCNTs and MWCNTs; however, the number of live cells was higher with MWCNTs.

Table 4.3: The cell viability results for haemocytes of *C. edule* exposed to different concentrations of different forms of CNTs measured by Trypan blue (n=3). The equivalent concentrations of 50µgL^{-1} = 0.1 µg.g^{-1}, 100µgL^{-1} = 0.2 µg.g^{-1} and 500µgL^{-1} =1 µg.g^{-1} in treatment 3. * significant differences between control or SRNOM and other concentration groups.

Concentration of CNTs	Trypan blue measurements					
	Treatment 1 (Water-spiked)		Treatment 2 (Surface-spiked)		Treatment 3 (Sediment-spiked)	
	SWCNT	**MWCNT**	**SWCNT**	**MWCNT**	**SWCNT**	**MWCNT**
Control	95%	96%	95%	97%	96%	95%
SRNOM-dispersed	94%	96%	95%	96%	95%	96%
50µgL^{-1}	88% *	91%	90%	92%	93%	95%
100µgL^{-1}	79% *	85% *	86% *	88% *	89% *	91%
500µgL^{-1}	76% *	80% *	78% *	84% *	83% *	85% *

4.3.2 Comet assay

In the present study, *C. edule* were exposed *in vivo* for 72 hours to nominal concentrations of 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} of SWCNTs or MWCNTs in treatment 1 and 2, and to equivalent concentrations of 0.1 µg.g^{-1} , 0.2 µg.g^{-1} and 1 µg.g^{-1} of SWCNTs or MWCNTs in treatment 3. As a result, the level of DNA damage is differentiated according to the differences between all treatments and concentrations. In all three treatments, no DNA damage was measured in the gill cells and haemocytes of the cockles exposed *in vivo* to Suwannee River natural organic matter (SRNOM) alone, compared to the control group in all treatments (Figures 4.11, 12 and 13).

In general, *C. edule* exposed *in vivo* to 50µg L^{-1} (or equivalent concentrations of 0.1 µg.g^{-1}) of SWCNTs and MWCNTs for 72 hours showed significantly increased DNA damage, which was measured in the gill cells and haemocytes only under treatment 1 (Figure 4.11). However, exposure of *C. edule* to 100µg L^{-1} and 500µg L^{-1} (or equivalent concentrations of 0.2 µg.g^{-1} and 1 µg.g^{-1}, respectively) of SWCNTs and MWCNTs showed significantly increased DNA strand breaks in both gill cells and haemocytes in all three treatments, as shown in Figures 4.11, 4.12 and 4.13. Gill cells were more sensitive than haemocytes in the cockles exposed to both SWCNTs and MWCNTs (one-way ANOVA, $P<0.05$, followed by the Tukey test). Furthermore, a statistical comparison was carried out between the levels of DNA damage in cockles after exposure to each form of CNT. The DNA damage was higher in *C. edule* cells exposed to SWCNTs than in the *C. edule* cells exposed to MWCNTs in all three treatments (Figures 4.11, 12 and 13).

In treatment 1 (water-spiked), the DNA damage resulting from exposing the cockles to 50µg L^{-1} of SWCNTs and MWCNTs is clear and it is significantly increased with increasing concentration. For example, in haemocytes, at a concentration of 100µg L^{-1}, the DNA damage is 12.6 % in SWCNTs and 10.6 % in MWCNTs, while at a concentration of 500µg L^{-1}, the DNA damage increased significantly to 22.8 %in SWCNTs and 19.1 %in MWCNTs, which shows an increase of 10.2% and 8.5% for SWCNT and MWCNT respectively (Figure 4.11).

In treatment 2 (surface-spiked), the DNA damage resulting from exposing the cockles to different concentrations of SWCNTs and MWCNTs is generally less than the DNA level damage of treatment 1. The DNA damage started to be noticeable at 50µg L^{-1}, while it significantly increased only in SWCNTs and MWCNTs 100µg L^{-1} and 500µgL^{1} compared to the control and SRNOM groups (Figure 4.12).

In treatment 3 (sediment spiked), the DNA damage level is less than that in treatments 1 and 2. There is a very little damage at 0.1 µg.g^{-1} in SWCNTs and it is not obvious in MWCNTs. However, there is still a significant increase in damage only at concentrations of 0.2 µg.g^{-1} and 1 µg.g^{-1} of both types of CNTs, compared to the control and SRNOM groups. Nevertheless, SWCNTs still show a more toxic effect and result in more DNA damage than MWCNTs – the level of DNA damage at a concentration of 1 µg.g^{-1} for SWCNTs and MWCNTs reached 13.1% and 9.7% respectively, measured in the gill cells (Figure 4.13).

Summary

- SRNOM had no significant effect on the DNA damage in the tail, as in the control.
- Gill samples showed an increase in DNA damage compared to haemocytes in all treatments.
- SWCNTs resulted in a higher percentage of DNA damage regardless of the type of treatment or concentration, compared to MWCNTs.
- Increasing CNT concentrations in all treatments resulted in increased DNA damage.
- Exposure of *C. edule* to 50µgL^{-1} in treatment 1 showed a significant increase in DNA damage only compared to control, while exposure of *C. edule* to 100 µgL^{-1}and 500 µgL^{-1} showed a significant increase in DNA damage in treatments 1 and 2. In treatment 3, the significant increase was shown at 0.2 µg.g^{-1} and 1.0 µg.g^{-1} only, regardless of the type of CNT.
- Treatment 1, with all the studied CNT concentrations, resulted in the highest DNA damage compared to treatments 2 and 3. Treatment 3 resulted in the lowest DNA damage when compared with treatments 1 and 2.

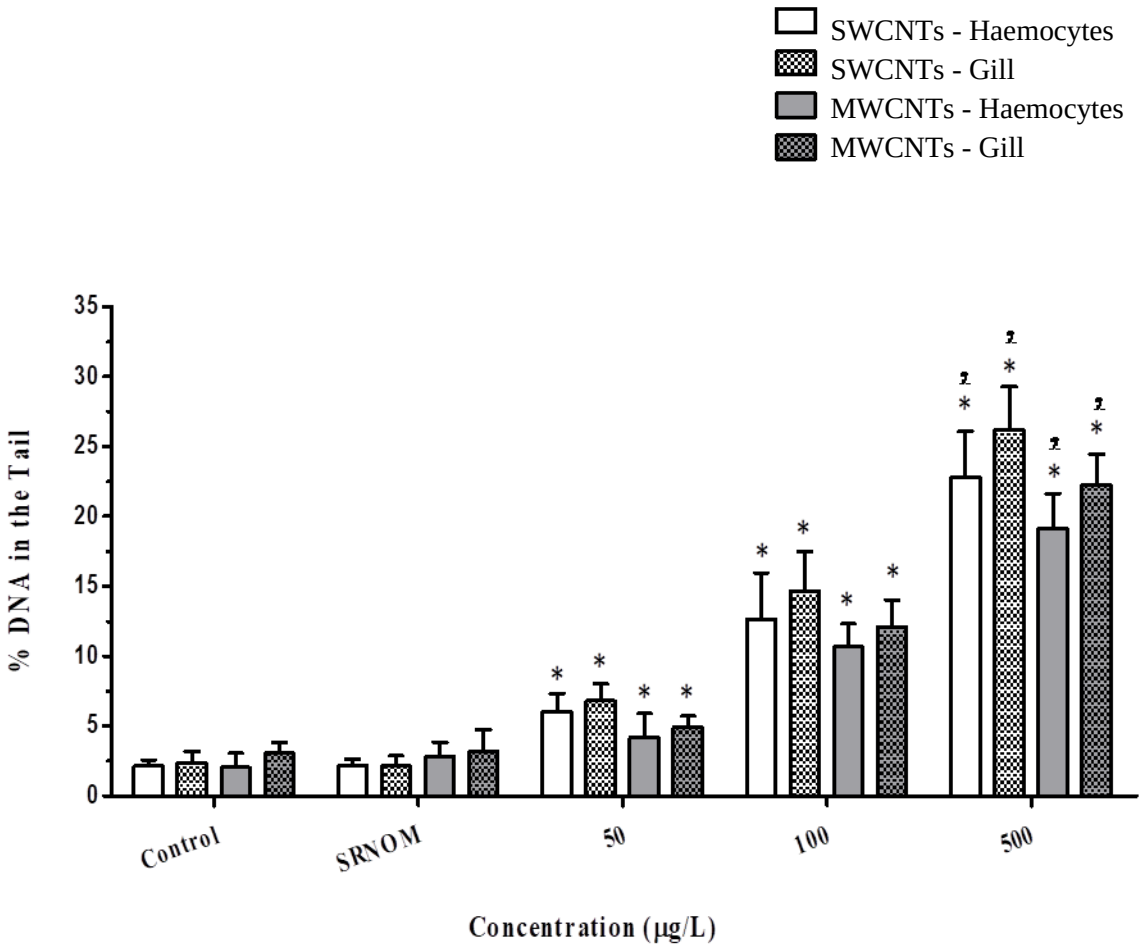


Figure 4.11: DNA damage, expressed as a percentage of DNA in the tail, in haemocytes and gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at different concentrations of 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} **under treatment 1 exposure condition**. A statistically significant increase in DNA damage was measured in haemocytes and gills. * significant differences between control or SRNOM and other concentration groups; ● significant differences between 500µg L^{-1} and other concentrations ($p<0.05$; mean ± standard deviation, n=3).

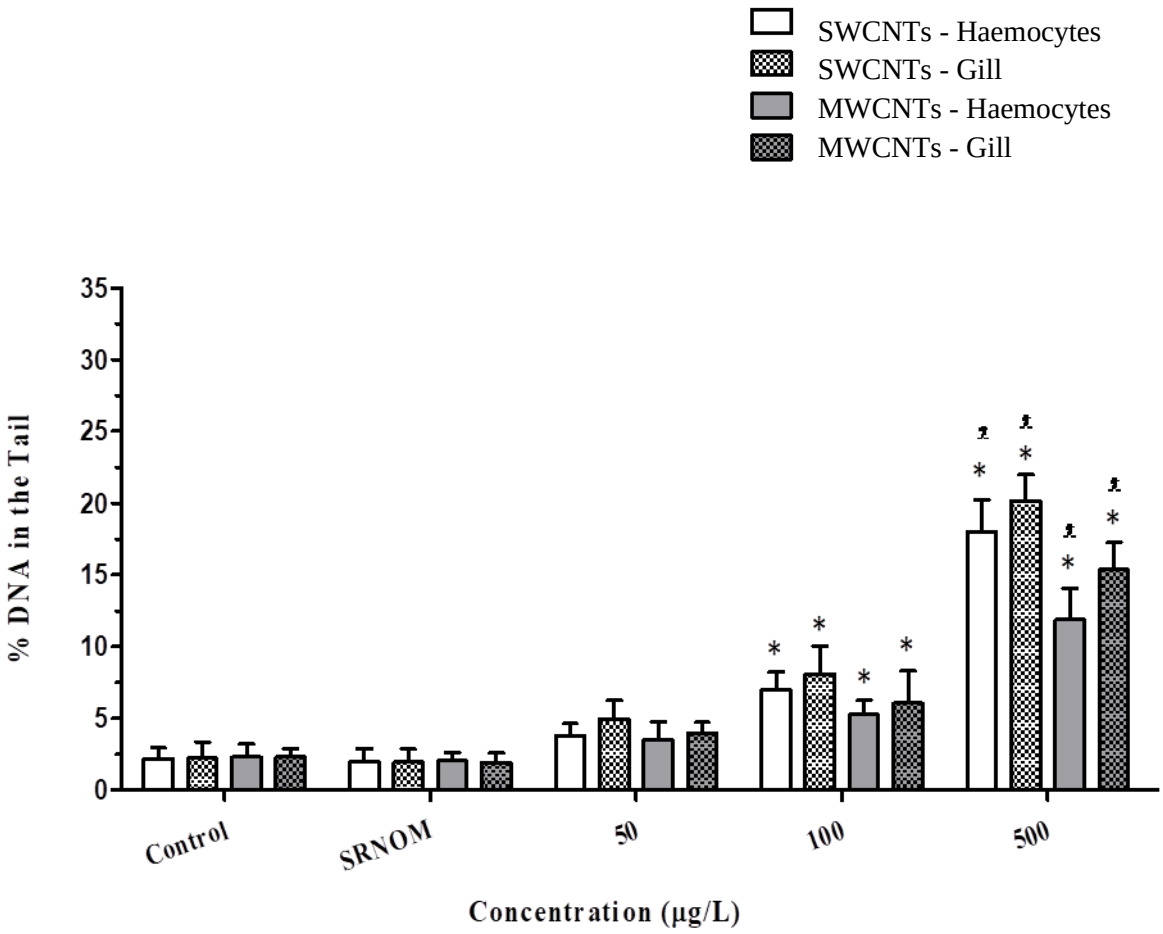


Figure 4.12: DNA damage, expressed as percentage of DNA in the tail, in haemocytes and gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at different concentrations of 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} **under treatment 2 exposure condition**. A statistically significant increase in DNA damage was measured in haemocytes and gills. * significantly different from control or SRNOM and other concentration groups; ♣ significant difference between 500µg L^{-1} and other concentrations ; ($p<0.05$; means ± standard deviation, n=3).

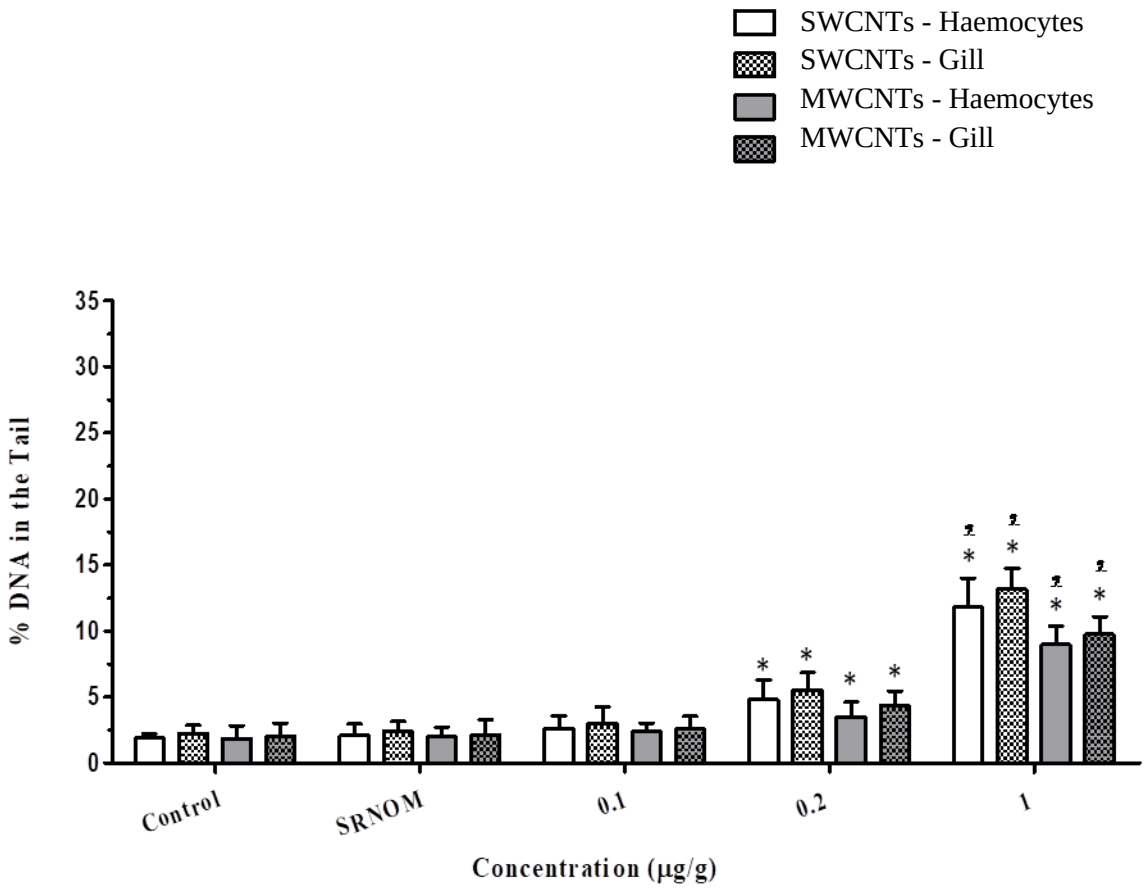


Figure 4.13: Results of DNA damage , expressed as percentage of DNA in the tail, in haemocytes and gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at equivalent concentrations of CNTs: 0.1 µg,g^{-1}, 0.2 µ$g.g^{-1}$ and 1 µ$g.g^{-1}$ **under treatment 3 exposure condition**. A statistically significant increase in DNA damage was measured in haemocytes and gills. * significantly different from control or SRNOM and groups with other concentrations; ♠ significant difference between 1 µ$g.g^{-1}$ and other concentrations; ($p<0.05$; means ± standard deviation, n=3).

4.3.3 Oxidative stress

4.3.3.1 Superoxide dismutase (SOD) and Thiobabituric acid reactive substances (TBARS)

Oxidative stress was measured by assessing the SOD activity and lipid peroxidation in the gills of cockles *C. edule*, using the SOD assay and TBARS assay, respectively. Therefore, oxidative stress in the gill cells of *C. edule in vivo* exposed to SWCNTs and MWCNTs for 72 hours, at three different concentrations: 50 μgL^{-1}; 100 μgL^{-1} and 500 μgL^{-1} under treatments 1 and 2 exposure conditions, and equivalent concentrations: 0.1 $\mu g.g^{-1}$, 0.2 $\mu g.g^{-1}$ and 1 $\mu g.g^{-1}$ under treatment 3 exposure condition. The results showed that there was a concentration-dependent increase in both SOD activity (expressed as a decrease in percentage inhibition) and lipid peroxidation (expressed as an increase in TBARS nMol mg $protein^{-1}$).

There is a significant increase in both SOD activity (Figure 4.14 and 4.15) and TBARS (Figure 4.17 and 4.18) in gill tissue from cockles exposed to both types of CNTs under treatments 1 and 2 exposure conditions. Although treatment 1 showed higher levels of SOD activity and TBARS than treatment 2, both treatments, water-spiked and surface-spiked, showed significantly higher SOD activity and TBARS for all concentrations and both CNT types, compared to the control or SRNOM. The SOD activity and TBARS was higher in SWCNTs than in MWCNTs.

In treatment 3, oxidative stress was also assessed at equivalent nominal concentrations of CNTs: 0.1 $\mu g.g^{-1}$, 0.2 $\mu g.g^{-1}$ and 1 $\mu g.g^{-1}$ spiked into the sediment. At concentrations of 0.1 $\mu g,g^{-1}$, the SOD activity level was not significantly different between each groups or when compared to the control group. However, the SOD activity was significantly increased at concentrations of 0.2 $\mu g.g^{-1}$ and 1 $\mu g.g^{-1}$ compared to the control or SRONM (Figure 4.16). Moreover, the levels of TBARS in gill cells were also significantly elevated compared to the control group or SRONM at concentrations of 1 $\mu g.g^{-1}$. In addition, a significant increase was observed at concentrations $\geq$ 0.2 $\mu g.g^{-1}$ compared to 0.1 $\mu g,g^{-1}$. No significant difference was observed between levels at 0.1 $\mu g,g^{-1}$ and SNORM or the control (Figure 4.19).

Summary

- Increasing SOD activity leads to an increase in oxidative stress and hence, decreases SOD % inhibition.
- SRNOM had no significant effect on the SOD % inhibition, TBARS or control.
- SWCNT had a lower SOD % inhibition, regardless of the type of treatment or concentration, compared to the MWCNT, which means that SWCNTs had higher SOD activity.
- Increasing CNT concentration in all treatments resulted in lower SOD % inhibition and higher TBARS.
- Exposure of cockles to 50μgL^{-1}, 100μgL^{-1} and 500 μgL^{-1} CNTs in treatments 1 & 2 did show significant decreases in SOD % inhibition or significant increases in lipid peroxidation (TBARS). However, in treatment 3, the

significant decrease of SOD % inhibition and significant increase in lipid peroxidation (TBARS) were shown only at 0.2 µg.g and 1.0µg.g, regardless of the type of CNTs.

- Treatment 1, with all the studied CNT concentrations, produced the lowest SOD % inhibition and the highest lipid peroxidation (TBARS) when compared to treatments 2 and 3. Treatment 3, where the CNTs was spiked into the sediment, resulted in the highest SOD % inhibition and lowest lipid peroxidation (TBARS).

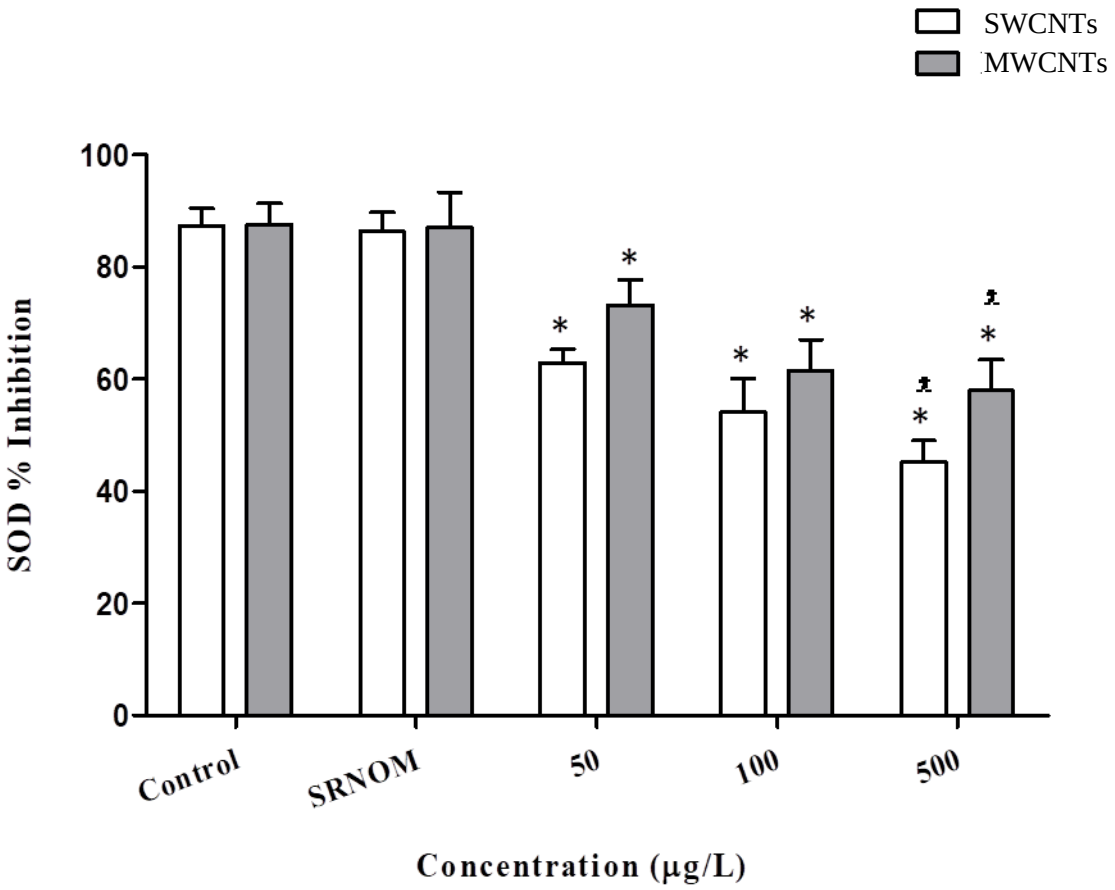


Figure 4.14: Superoxide dismutase activity (expressed as percentage of inhibition) in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at different concentrations: 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} **under treatment 1 exposure condition**. * significant differences between control or SRNOM and other concentration groups; ♣ significant difference between 500µg L^{-1} and other concentrations; ($p<0.05$; means ± standard deviation, n=3).

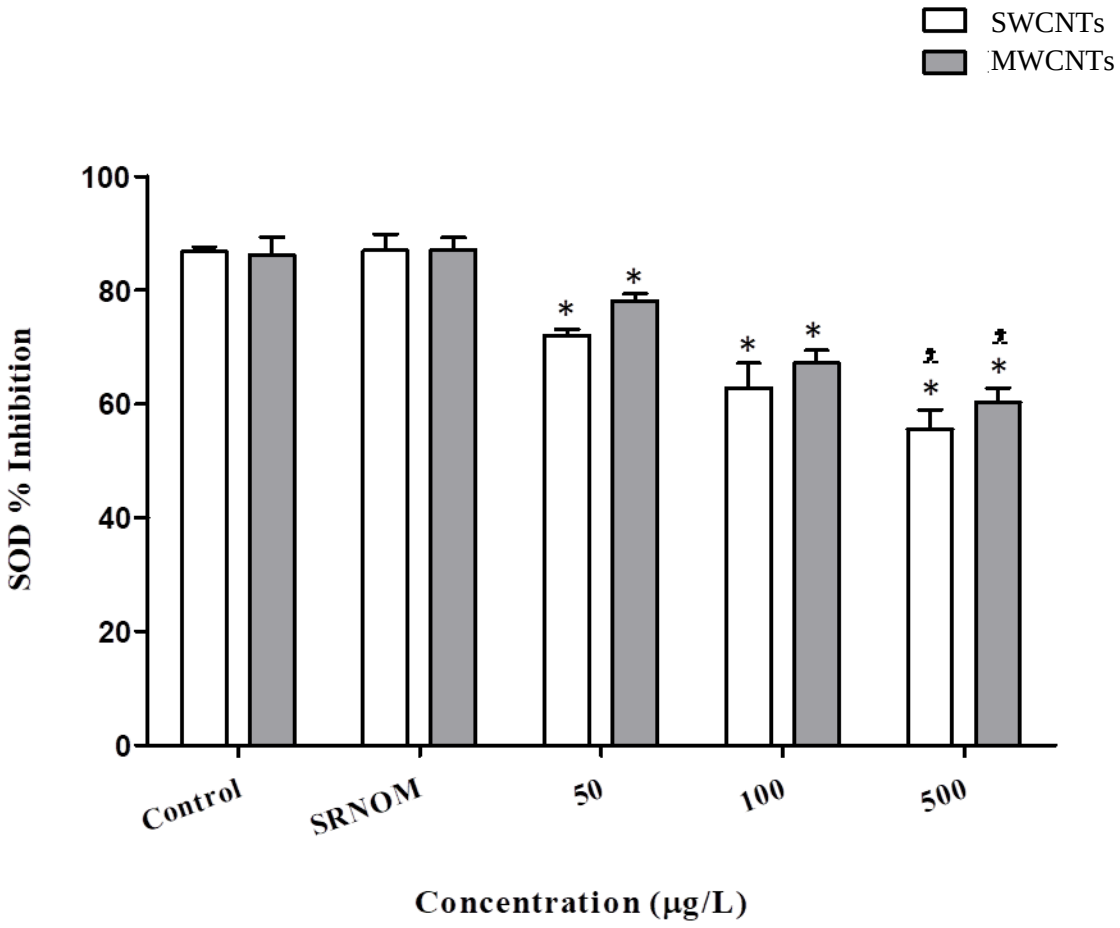


Figure 4.15: Superoxide dismutase activity (expressed as percentage of inhibition) in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at different concentrations, 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} **under treatment 2 exposure condition**. * significant differences between control or SRNOM and other concentration groups; ♣ significant difference between 500µg L^{-1} and other concentrations; ($p<0.05$; means ± standard deviation, n=3).

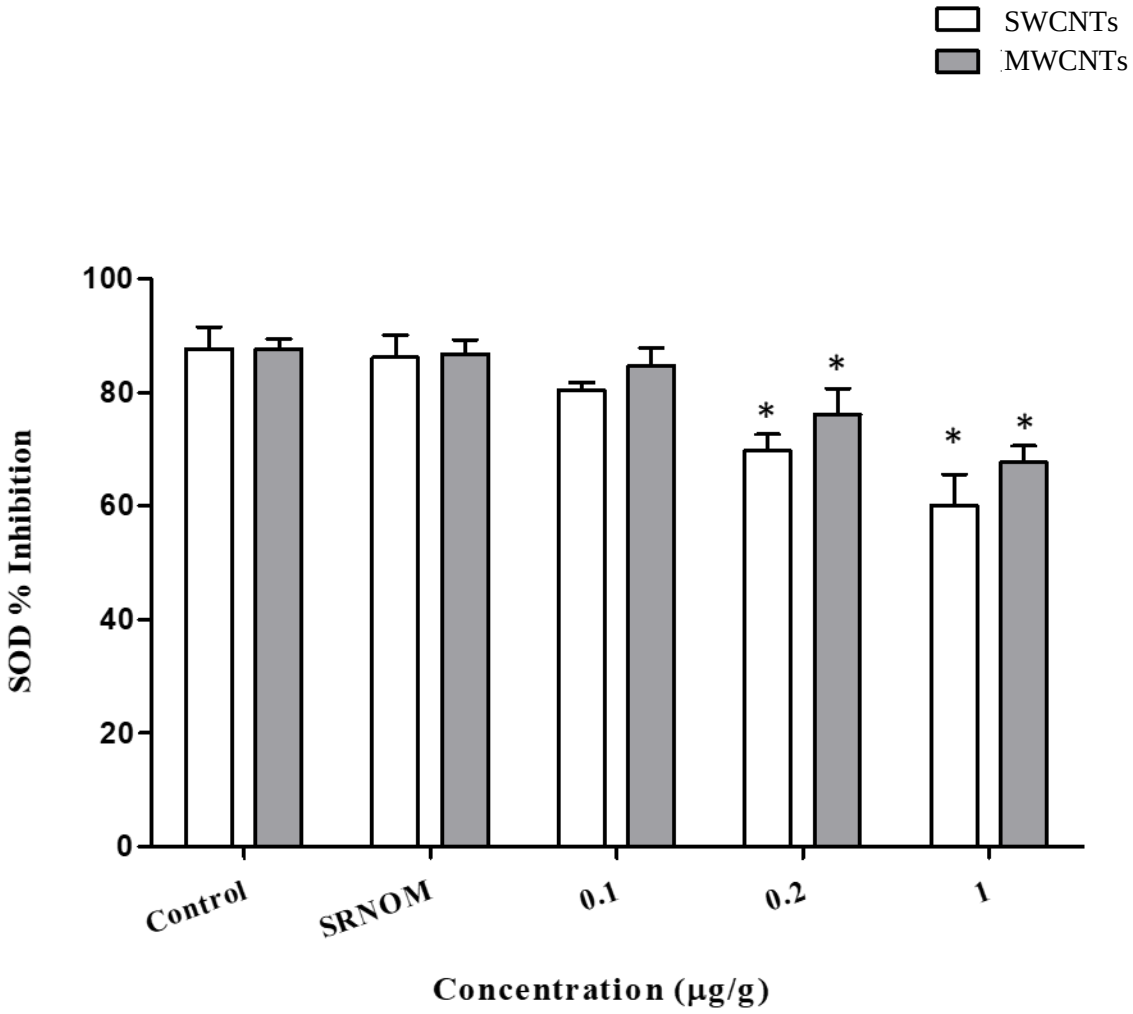


Figure 4.16: Superoxide dismutase activity (expressed as percentage of inhibition) in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at equivalent concentrations of CNTs: 0.1 µg,g^{-1}, 0.2 µg.g^{-1} and 1 µg.g^{-1} **under treatment 3 exposure condition**. * significant differences between control or SRNOM and other concentration groups; ♣ significant difference between 1 µg.g^{-1} and other concentrations; ($p<0.05$; means ± standard deviation, n=3).

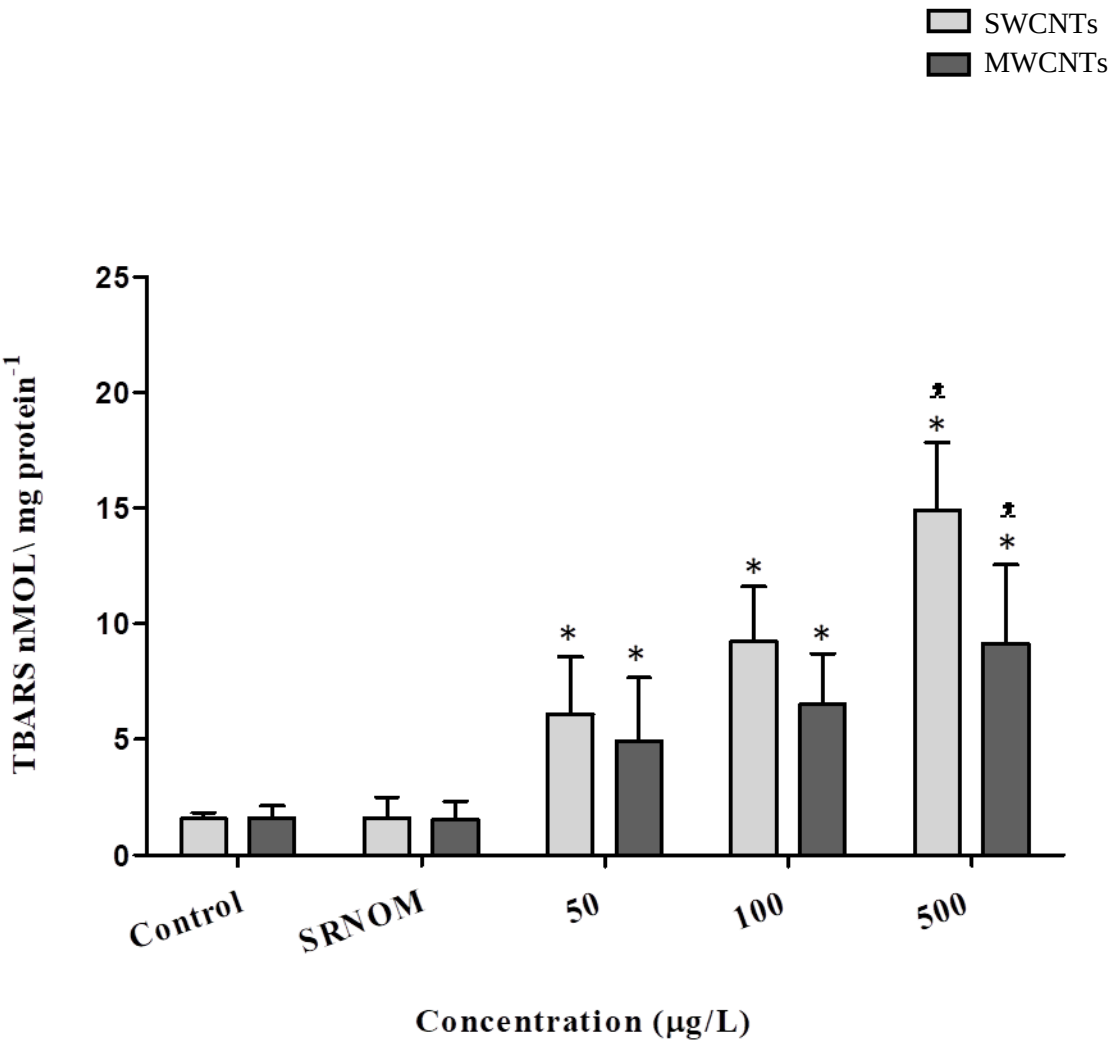


Figure 4.17: Amount of thiobarbituric acid reactive substances, expressed as nMol TBARS per mg protein, in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at different concentrations: 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} **under treatment 1 exposure condition**. * significant differences between control or SRNOM and other concentration groups; ⁂ significant differences between 500µg L^{-1} and other concentrations; ($p<0.05$; means ± standard deviation, n=3).

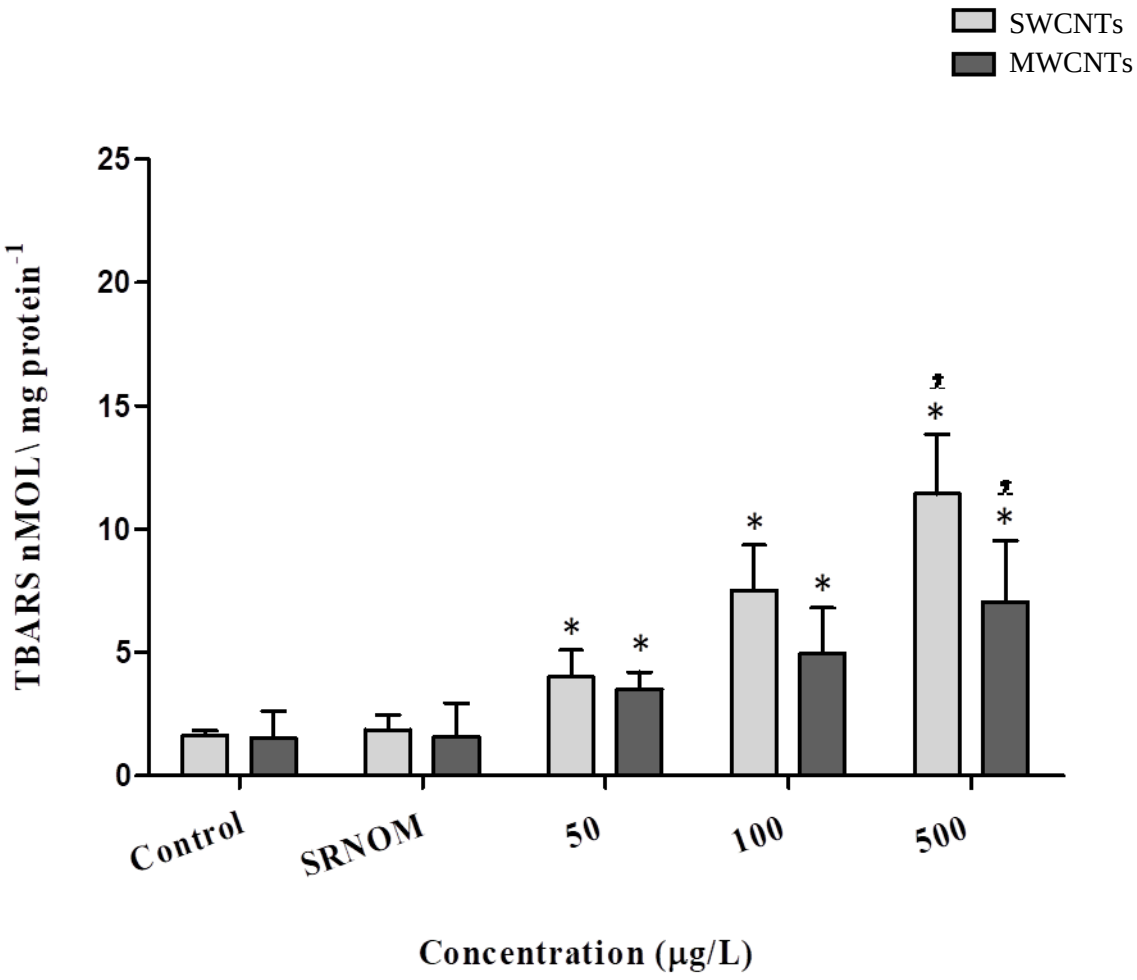


Figure 4.18: Amount of thiobarbituric acid reactive substances, expressed as nMol TBARS per mg protein, in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at different concentrations: 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} **under treatment 2 exposure condition**. * significant differences between control or SRNOM and other concentration groups; ♠ significant difference between 500µg L^{-1} and other concentrations; ($p<0.05$; means ± standard deviation, n=3).

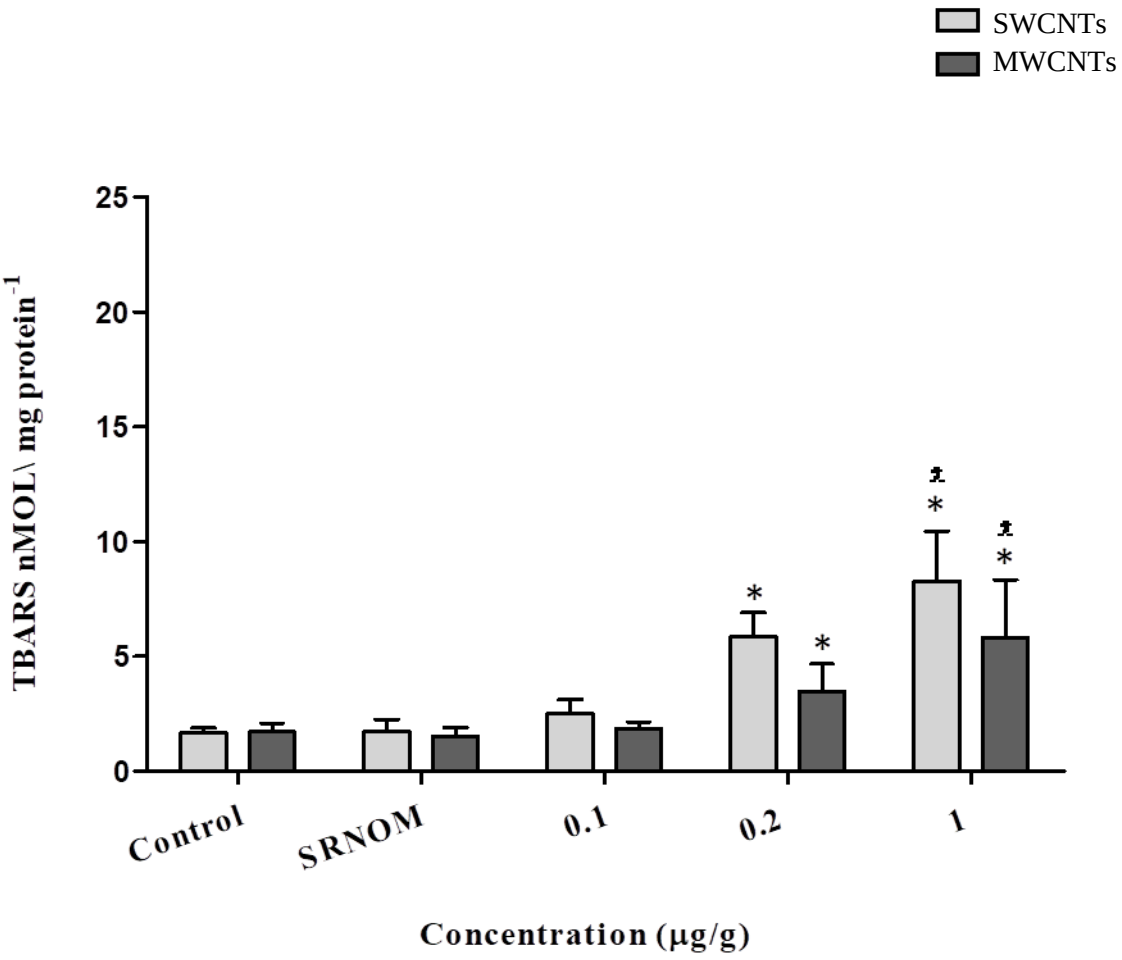


Figure 4.19: Amount of thiobarbituric acid reactive substances, expressed as nMol TBARS per mg protein, in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at different equivalent concentrations of CNTs: 0.1 µg.g^{-1}, 0.2 µg.g^{-1} and 1 µg.g^{-1} **under treatment 3 exposure condition**. * significant differences between control or SRNOM and other concentration groups; ♣ significant differences between 1 µg.g^{-1} and other concentrations; ($p<0.05$; means ± standard deviation, n=3).

4.4 The Effect of CNTs on the Bioavailability of Sediment-Associated Contaminants

4.4.1 Dynamic light scattering (DLS) and zeta potential within sediment-associated contaminants

The agglomerates for both types of CNTs in combination with dissolved metals Cd and Zn under exposure conditions were still negatively charged: the SWCNTs' charge with **Cd** 0.001µM ranged between -2.85 and -8.64 mV, while that of the MWCNTs ranged between -4.31 and $^{-1}$0.52 mV (Table 4.4). Meanwhile, the SWCNTs' charge with **Zn** 1.0µM ranged between -3.25 and -7.45 mV, while that of the MWCNTs ranged between -5.15 and -8.22 mV (Table 4.4). In combination with dissolved metals, the approximate sizes for both types of CNTs were found to increase in a concentration-dependent manner (Table 4.4). For example, in combination with **Cd** 0.001µM, at the concentration of 50 µg L^{-1}, the SWCNTs' agglomerate length was 964.66 nm while that of the MWCNTs was 1305.5 nm.

Table 4.4: Average agglomerate size and zeta potential of SWCNT and MWCNT particulates, in the presence of dissolved metals, at different concentrations suspended in seawater (under exposure conditions), measured by DLS.

CNT Types	CNT (µg L^{-1})	DLS (nm)		Zeta potential (seawater)	
		Cd 0.001µM	**Zn** 1.0µM	**Cd** 0.001µM	**Zn** 1.0µM
SWCNTs	**50**	964.66	812.32	-2.85	-3.25
	100	3250.33	2945.33	-5.51	-4.91
	500	5322.45	5163.45	-8.64	-7.45
MWCNTs	**50**	1305.5	1503.42	-4.31	-5.15
	100	4010.3	3942.5	-7.34	-8.52
	500	3731.63	4511.54	-10.52	-8.22

pH 8.4, salinity 32 (±1) ‰

DLS = dynamic light scattering

4.4.2 Interaction between CNTs and Dissolved Metals

Tables 4.5 and 4.6 show CNTs' observed metal scavenging behaviour with Cd^{+2} and Zn^{+2} under the exposure conditions. Calibration of Cd^{+2} (Figure 4.20) and Zn^{+2} (Figure 4.21) standard curves are shown.

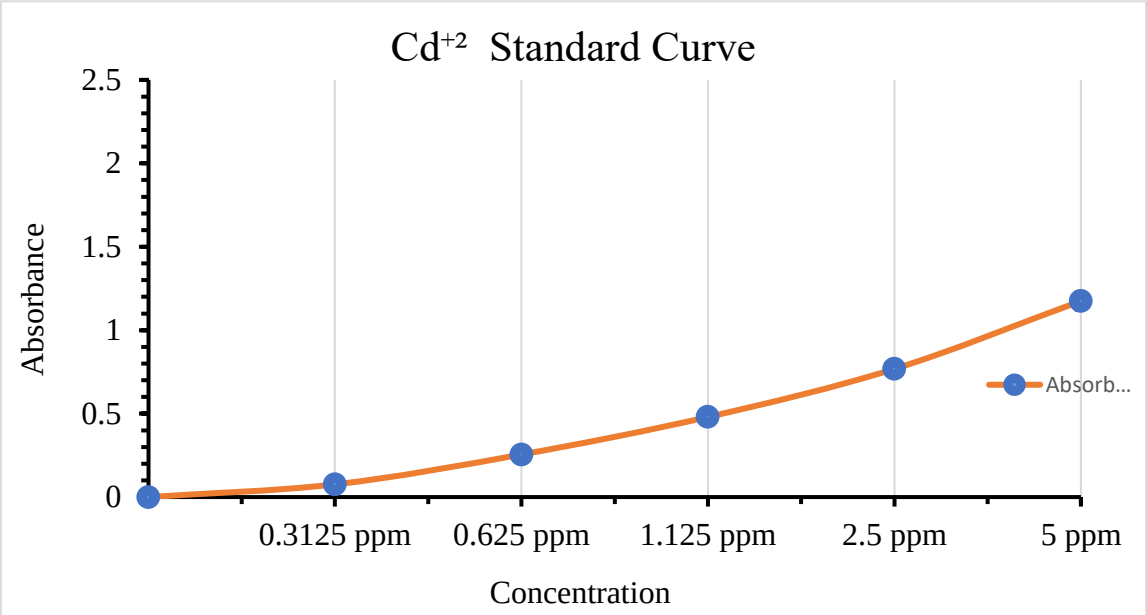


Figure 4.20: Calibration of Cd^{+2} standard curve between absorbance and concentration

Table 4.5: Average of chemical analysis of absorbance and recovery for CNTs with the Cd concentration in supernatant and in pellet (n=3)

Cadmium nominal	**post-Centrifugation**	**Cd measured (n=3)**		**Recovery %**	
		SWCNTs	MWCNTs	SWCNTs	MWCNTs
3.16 ppm	**Supernatant**	0.98 ppm	0.56 ppm	31.01 %	17.72 %
	Pellet	1.97 ppm	2.51 ppm	62.24 %	79.43 %

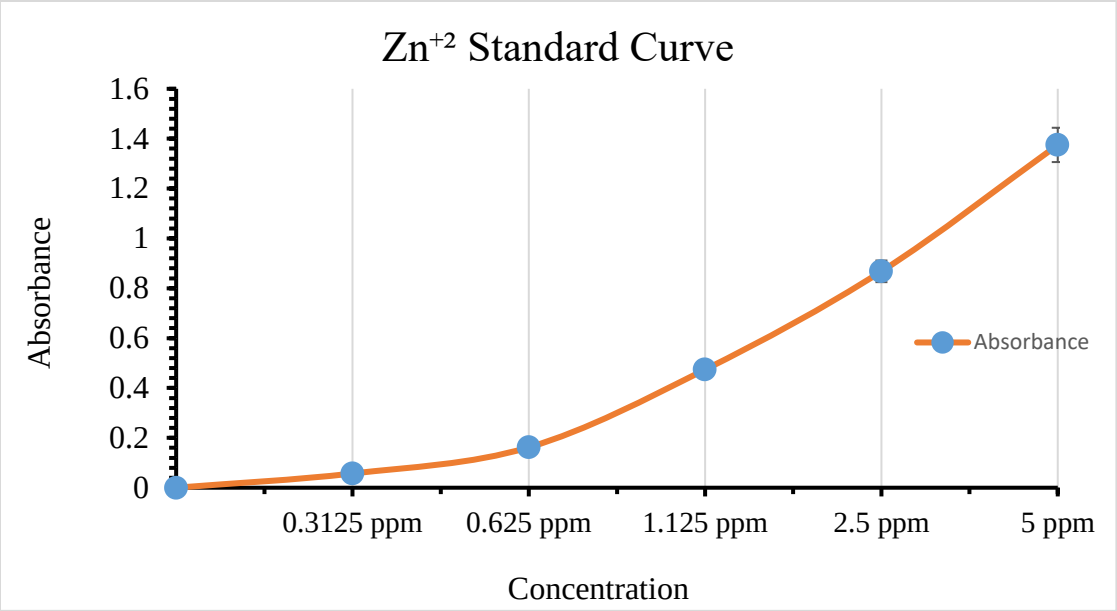


Figure 4.21: Calibration of Zn^{+2} standard curve between absorbance and concentration

Table 4.6: Average of chemical analysis of absorbance and recovery for CNTs with the Zn concentration in supernatant and in pellet (n=3).

Zinc nominal	post-Centrifugation	Zn measured (n=3)		Recovery %	
		SWCNTs	MWCNTs	SWCNTs	MWCNTs
6.45 ppm	**Supernatant**	2.15 ppm	1.20 ppm	33.3 %	18.6 %
	Pellet	4.12 ppm	4.94 ppm	63.8 %	76.5 %

4.4.3 CNT and sediment-associated contaminant Interaction within Cockles

The results showed that the amount of Cd^{+2} and Zn^{+2} accumulated by the cockles increased when the metals interacted with both forms of CNTs (Table 4.7).

Table 4.7: Chemical analysis of metals (Cd^{2+} and Zn^{2+}) in gills. (n=3). Mean ± SD.

Chemical Exposure	Cd^{+2} in cockles' gills (ppm)	Zn^{+2} in cockles' gills (ppm)
Control Sedimnets	1.323 ± 3.2	51.521 ± 2.9
Control Cockles Sample	0.421 ± 1.2	34.003 ± 2.2
SWCNTs 100 μg L^{-1}	0.656 ± 1.5	34.862 ± 1.7
MWCNTs 100 μg L^{-1}	0.736 ± 1.7	35.060 ± 2.1
Cd^{+2}	1.097 ± 0.5	35.173 ± 0.9
Zn^{+2}	0.597 ± 1.1	35.756 ± 2.3
SWCNTs 100 μg L^{-1} + Cd^{+2}	1.438 ± 2.2	34.924 ± 1.9
SWCNTs 100 μg L^{-1} + Zn^{+2}	0.583 ± 1.4	37.258 ± 0.8
SWCNTs 100 μgL^{-1} + Cd^{+2} + Zn^{+2}	1.287 ± 0.4	36.033 ± 1.1
MWCNTs 100 μg L^{-1} + Cd^{+2}	1.948 ± 1.8	34.744± 1.2
MWCNTs 100 μgL^{-1} + Zn^{+2}	0.530 ± 0.8	38.782 ± 0.5
MWCNTs 100 μgL-1 + Cd^{+2} + Zn^{+2}	1.644 ± 1.1	37.163 ± 1.4

4.4.4 The toxicity of CNTs with sediment-associated contaminants

In order to study the effect of sediment-associated contaminants (Cd and Zn) in the presence of each type of CNT, *C. edule* was first exposed to both SWCNTs and MWCNTs at concentrations of 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} mixed with metals Cd 0.001µM and Zn 1µM under treatments 1 and 2 exposure condition. Then, *C. edule* was exposed to both SWCNTs and MWCNTs at equivalent concentrations of 0.1 µg.g^{-1}, 0.2 µg.g^{-1} and 1 µg.g^{-1} under treatment 3 exposure conditions, and contaminated with two different types of sediment associated contaminants, Cd 0.001µM and Zn 1µM for 72 hours. The following sections present the results obtained from the different biomarker tests used.

4.4.4.1 Cell viability

The Trypan blue approach expresses the percentage of live and dead cells under the light microscope. The dead cells appear blue by taking up the blue dye, while the live cells appear normal. The cell viability results obtained from the Trypan blue technique showed that cell viability decreased in the haemocytes of *C. edule* when exposed to CNTs separately and in combination with metals for 72 hours. Tables 4.8, 4.9 and 4.10 show that the cell viability decreased in a concentration-dependent manner. Moreover, Tables 4.8, 4.9 and 4.10 show that there were significant differences between the percentage of live cells when exposed to metals (Cd or Zn) and in the presence of either SWCNTs or MWCNTs under all three treatment conditions, although the number of live cells in SWCNTs was higher.

Table 4.8: The cell viability results for haemocytes of *C. edule* exposed to different forms of CNTs with or without Zn and Cd at 50µg L^{-1} in treatments 1 & 2 or equivalent concentrations of CNTs (0.1 µg.g^{-1}) in treatment 3 (n=3). SW = SWCNTs, MW = MWCNTs. * significant differences between control or SRNOM and other concentration groups.

CNT Concentration of 50µg L^{-1}	**Trypan blue measurements**					
	Treatment 1 (Water-spiked)		Treatment 2 (Surface-spiked)		Treatment 3 (Sediment-spiked)	
	SW	**MW**	**SW**	**MW**	**SW**	**MW**
Control	96%	94%	95%	96%	95%	97%
Cd 0.001µM	95%	95%	96%	95%	96%	96%
Zn 1.0 µM	97%	98%	94%	96%	95%	94%
Cd 0.001µM + Zn 1.0 µM	94%	96%	95%	95%	94%	96%
50µg L^{-1} CNTs	88% *	91%	90%	92%	93%	95%
50µg L^{-1} + Cd 0.001µM	83% *	81% *	87% *	85% *	90%	89% *
50µg L^{-1} + Zn 1.0µM	82% *	81% *	88% *	86% *	90% *	89% *
50µg L^{-1} + Cd 0.001µM + Zn 1.0µM	80% *	79% *	85% *	85% *	87% *	84% *

Table 4.9: The cell viability results for haemocytes of *C. edule* exposed to different forms of CNTs, with or without Zn and Cd, at 100µg L^{-1} in treatments 1 & 2 or equivalent concentrations of CNTs: (0.2 µg.g^{-1}) in treatment 3 (n=3). SW = SWCNTs, MW = MWCNTs. * significant differences between control or SRNOM and other concentration groups.

CNTs Concentration of 100µg L^{-1}	**Trypan blue measurements**					
	Treatment 1 (Water-spiked)		Treatment 2 (Surface-spiked)		Treatment 3 (Sediment-spiked)	
	SW	**MW**	**SW**	**MW**	**SW**	**MW**
Control	96%	94%	95%	96%	95%	97%
Cd 0.001µM	95%	95%	96%	97%	96%	97%
Zn 1.0 µM	96%	95%	93%	97%	95%	94%
Cd 0.001µM + Zn 1.0 µM	95%	96%	94%	96%	95%	94%
100µg L^{-1} CNTs	79% *	85% *	86% *	88% *	89% *	91%
100µg L^{-1} + Cd 0.001µM	75% *	79% *	79% *	83% *	84% *	84% *
100µg L^{-1} + Zn 1.0µM	78% *	82% *	83% *	81% *	83% *	84% *
100µg L^{-1} + Cd 0.001µM + Zn 1.0µM	72% *	75% *	78% *	77% *	82% *	80% *

Table 4.10: The cell viability results for haemocytes of *C. edule* exposed to different forms of CNTs, with or without Zn and Cd, at 500µg L^{-1} in treatments 1 & 2 or equivalent concentrations of CNTs: (1 µg.g^{-1}) in treatment 3. (n=3). SW = SWCNTs, MW = MWCNTs. * significant differences between control or SRNOM and other concentration groups.

CNTs Concentration of 500µg L^{-1}	**Trypan blue measurements**					
	Treatment 1 (Water-spiked)		Treatment 2 (Surface-spiked)		Treatment 3 (Sediment-spiked)	
	SW	**MW**	**SW**	**MW**	**SW**	**MW**
Control	96%	94%	95%	96%	95%	97%
Cd 0.001µM	95%	95%	96%	97%	96%	97%
Zn 1.0 µM	97%	95%	95%	96%	95%	97%
Cd 0.001µM + Zn 1.0 µM	96%	95%	95%	94%	95%	96%
500µg L^{-1}	77% *	81% *	78% *	84% *	84% *	86% *
500µg L^{-1} + Cd 0.001µM	75% *	79% *	76% *	83% *	83% *	83% *
500µg L^{-1} + Zn 1.0µM	75% *	80% *	77% *	80% *	81% *	84% *
500µg L^{-1} + Cd 0.001µM + Zn 1.0µM	61% *	63% *	71% *	70% *	76% *	76% *

4.4.5.2 Comet assay

The results of the comet assay are presented in Figures 4.22-4.24 (A, B and C). From these results, it was noticed that the exposure of *C. edule* to 0.001µM $CdCl_2$ had no effect on DNA damage when compared to the control. Even though Zn 1µM is less genotoxic than Cd 0.001µM, its effect was similar to that of Cd 0.001µM alone. In the same way, the Cd 0.001µM + Zn 1µM mixture did not increase DNA damage in *C. edule*'s haemocytes or gill cells.

DNA damage resulting from exposing the cockles to ≥50µg L^{-1} (or ≥0.1 µg.g^{-1} equivalent concentration in treatment 3) dispersed-SWCNTs or MWCNTs combined with Cd 0.001µM and Zn 1µM, separately, was significantly higher in all treatments, when compared to that resulting from exposure to metals alone. The level of damage was further significantly increased when Cd 0.001µM and Zn 1µM were both combined with the CNTs, when compared to that resulting from exposure to CNTs alone. For example, in treatment 2 (Figure 4.22B), while the level of DNA damage caused by SWCNTs in the haemocytes of the cockle was 3.8%, it increased to 6.1% and 5.0% when the cockle was exposed to Cd 0.001µM + SWCNTs or Zn 1µM + SWCNTs respectively. The DNA damage was further increased to 12.7 % when the cockle was exposed to Cd 0.001µM + Zn 1µM + SWCNTs.

It is clear there are differences between the three treatments with exposure to ≥50µgL^{-1}, or equivalent concentrations ≥0.1 µg.g^{-1}, CNTs. For example, in treatment 1 (Figure 4.22A), the level of DNA damage to the gill of the cockle exposed to Cd 0.001µM +

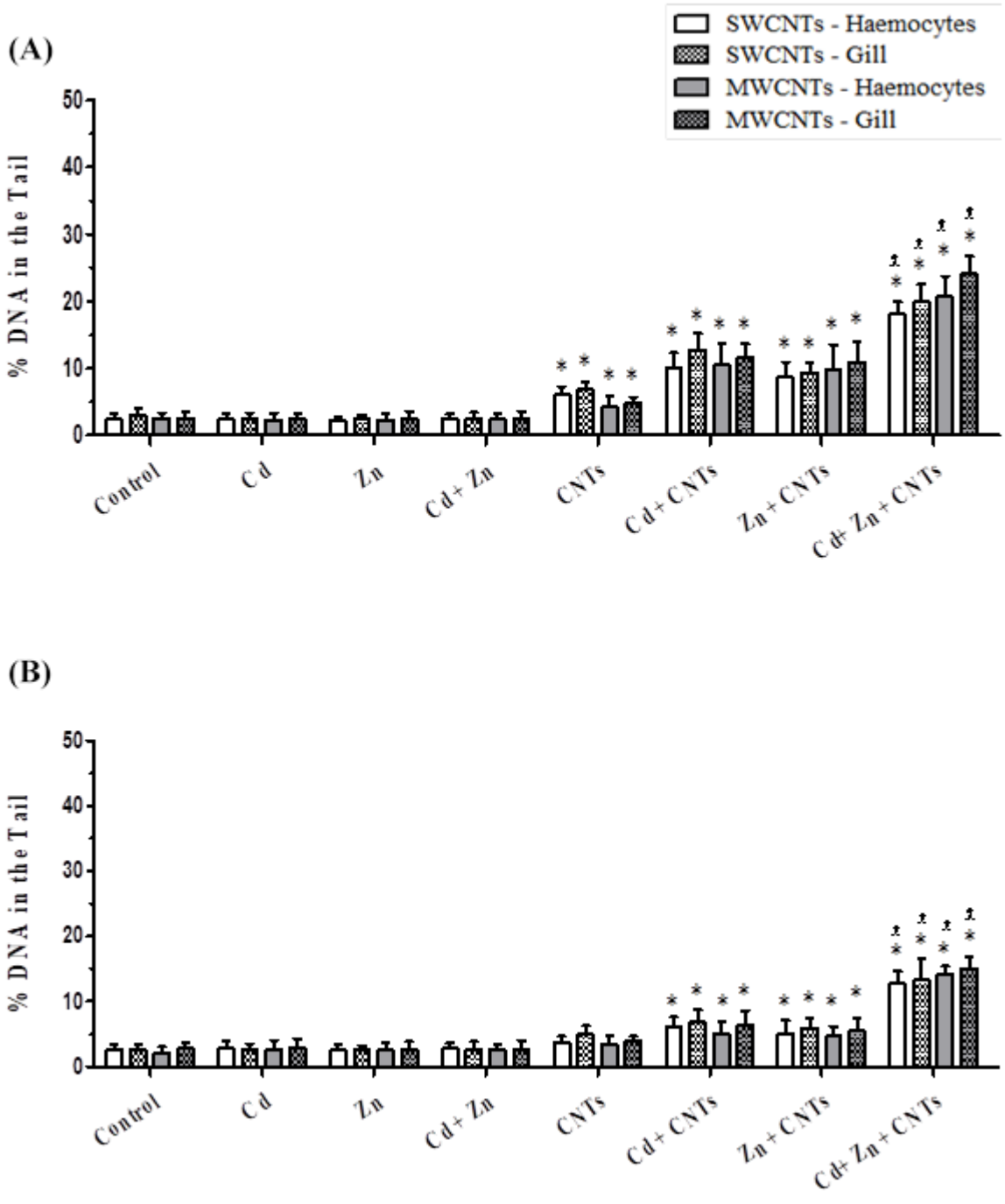
Zn 1µM + SWCNTs was 19.98%, while in treatment 2 (Figure 4.22B), it was only 13.32%, and in treatment 3 (Figure 4.22C) it was 12.64%. Thus, treatment 1 incurs greater DNA damage than treatment 2 and treatment 3, which incurred the lowest DNA damage.

Interestingly, when adding the metals to the dispersed-SWCNT or MWCNT, it was noticed that in all the treatments there were differences in the level of DNA damage. Furthermore, the DNA damage in the dispersed-MWCNT was almost equal to that in the dispersed-SWCNTs when they were mixed with the sediment-associated contaminants, which means that when the sediment-associated contaminants were added, the DNA damage difference with MWCNTs was higher than with SWCNTs, compared to the CNTs alone. For example, in treatment 2 (Figure 4.22B), where the cockle was exposed to 50µg L^{-1} SWCNTs alone, the DNA damage in the haemocyte measured 3.8%, while when it was exposed to Cd 0.001µM + Zn 1µM + 50µg L^{-1} SWCNTs, the level of damage was 12.7%, giving a difference of 8.9%. On the other hand, for the cockle exposed to 50µg L^{-1} MWCNTs alone, the DNA damage in the haemocyte was 3.4%, but when it was exposed to Cd 0.001µM + Zn 1µM + 50µg L^{-1} MWCNTs, the level of damage jumped to 14.1%, a greater increase of 10.7% (Figure 4.22B). A similar pattern can be observed with exposure to 100µg L^{-1} (Figure 4.23) and 500µg L^{-1} (Figure 4.24). The only difference is the overall increase of DNA damage at higher concentrations.

Summary

- Increasing the concentration of CNTs, when they are associated with metals, results in increased DNA damage, in all treatments.
- Cd 0.001µM, Zn 1.0µM and Cd 0.001µM + Zn 1.0µM had no significant effect on the DNA damage in the tail when compared to the control.
- Gill samples showed a greater increase in DNA damage compared to haemocytes.
- DNA damage increased significantly when SWCNTs and MWCNTs were contaminated with metals, in all treatments.
- DNA damage in cockles exposed to MWCNTs increased and became almost equal to the DNA damage resulting from SWCNTs when they were also exposed to metals.
- The increase in DNA damage from exposure to MWCNTs was higher than that from exposure to SWCNTs, in the presence of metals.
- For all the studied CNT concentrations contaminated with metals, treatment 1 resulted in the highest DNA damage when compared to treatment 2 and treatment 3; the latter resulted in the least DNA damage.
- The DNA damage was further increased when the cockle was exposed to Cd 0.001µM + Zn 1µM + SWCNTs, in all three treatments.

DNA damage in Cockles (50 µgL^{-1} CNTs)



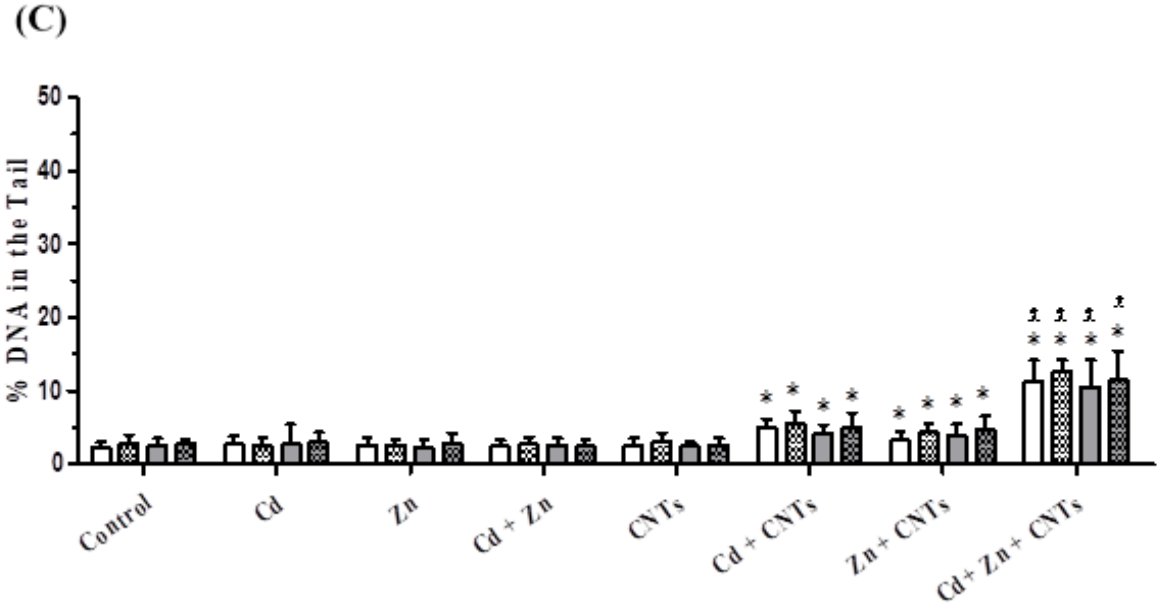
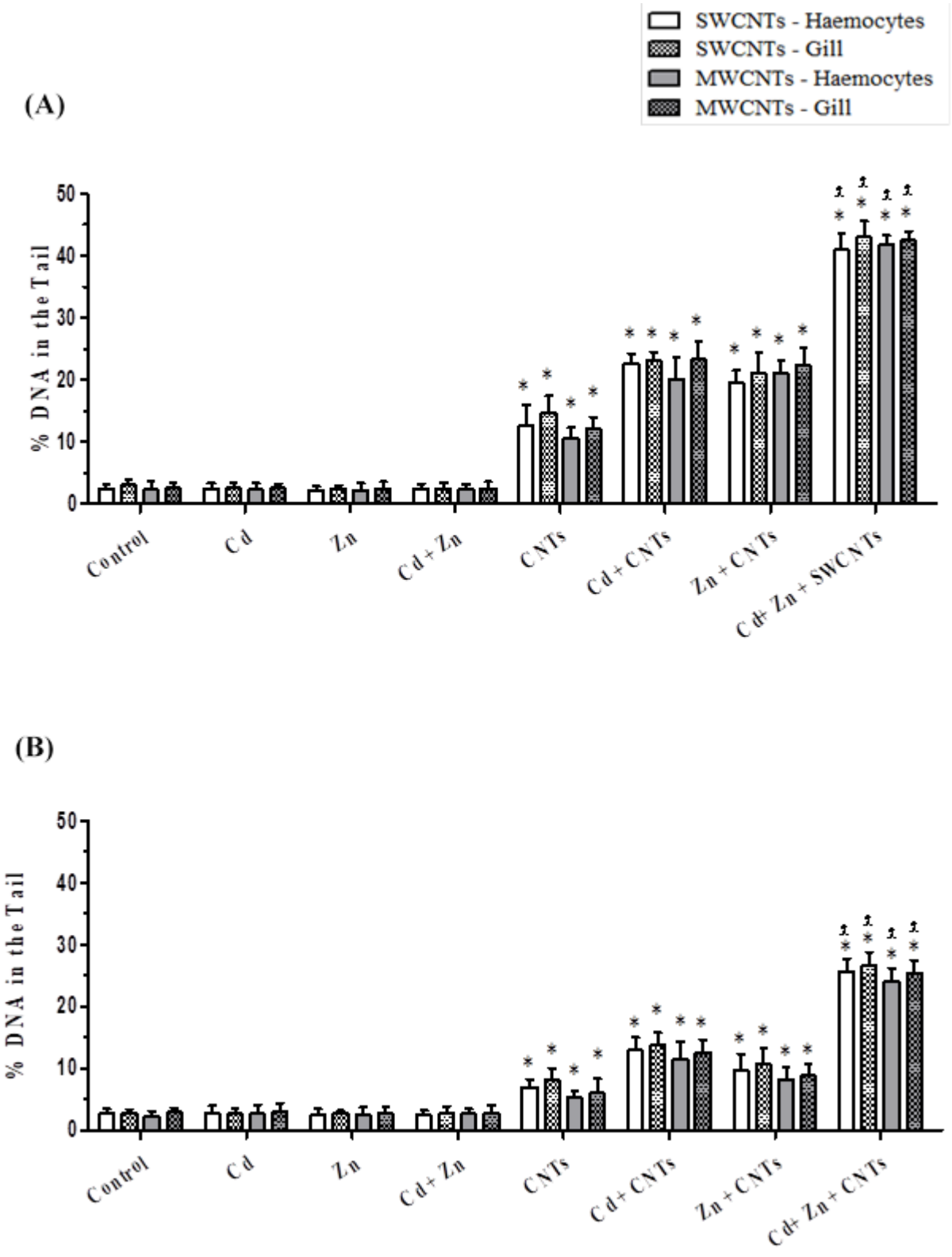


Figure 4.22: DNA damage, expressed as percentage of DNA in the tail, haemocytes and gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at 50µg L^{-1} alone, 50µg L^{-1} + **Cd** 0.001µM, 50µg L^{-1} + **Zn** 1.0µM and 50µg L^{-1} + **Cd** 0.001µM, + **Zn** 1.0µM: In treatment 1 (A) and treatment 2 (B); or equivalent concentrations of CNTs (0.1 µg.g^{-1}) in treatment 3 (C). In all three treatments, statistically significant increases in DNA damage were measured in haemocytes and gills. * significantly different from control, and from Cd, Zn and Cd + Zn; • significantly different from CNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=3).

DNA damage in Cockles (100 µgL^{-1} CNTs)



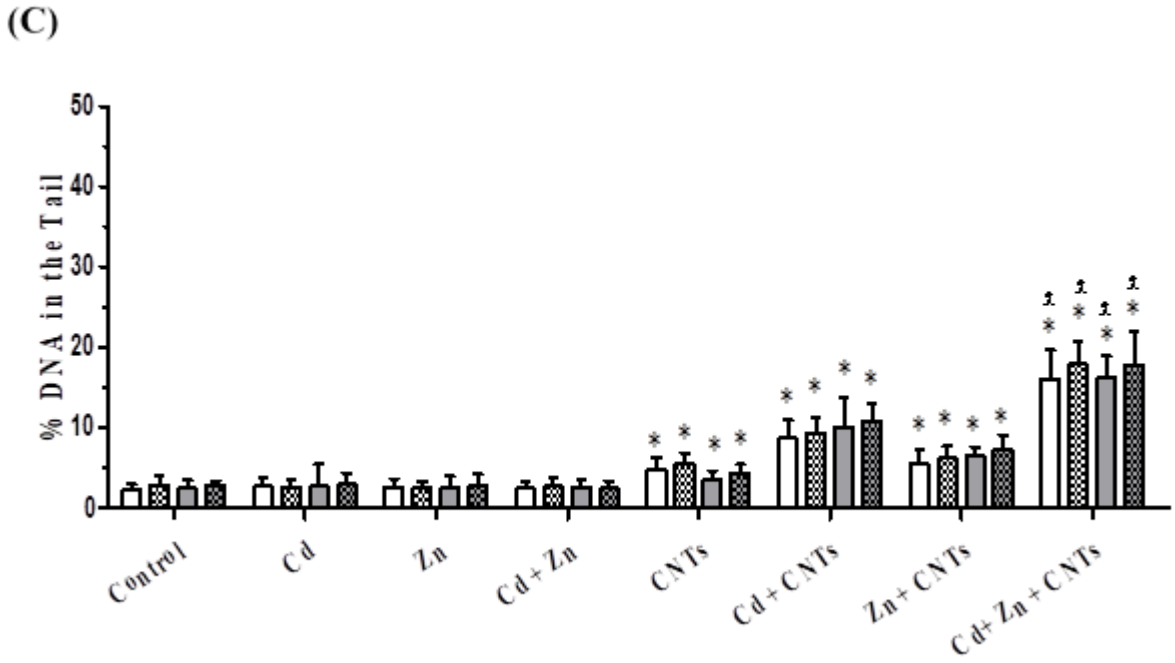
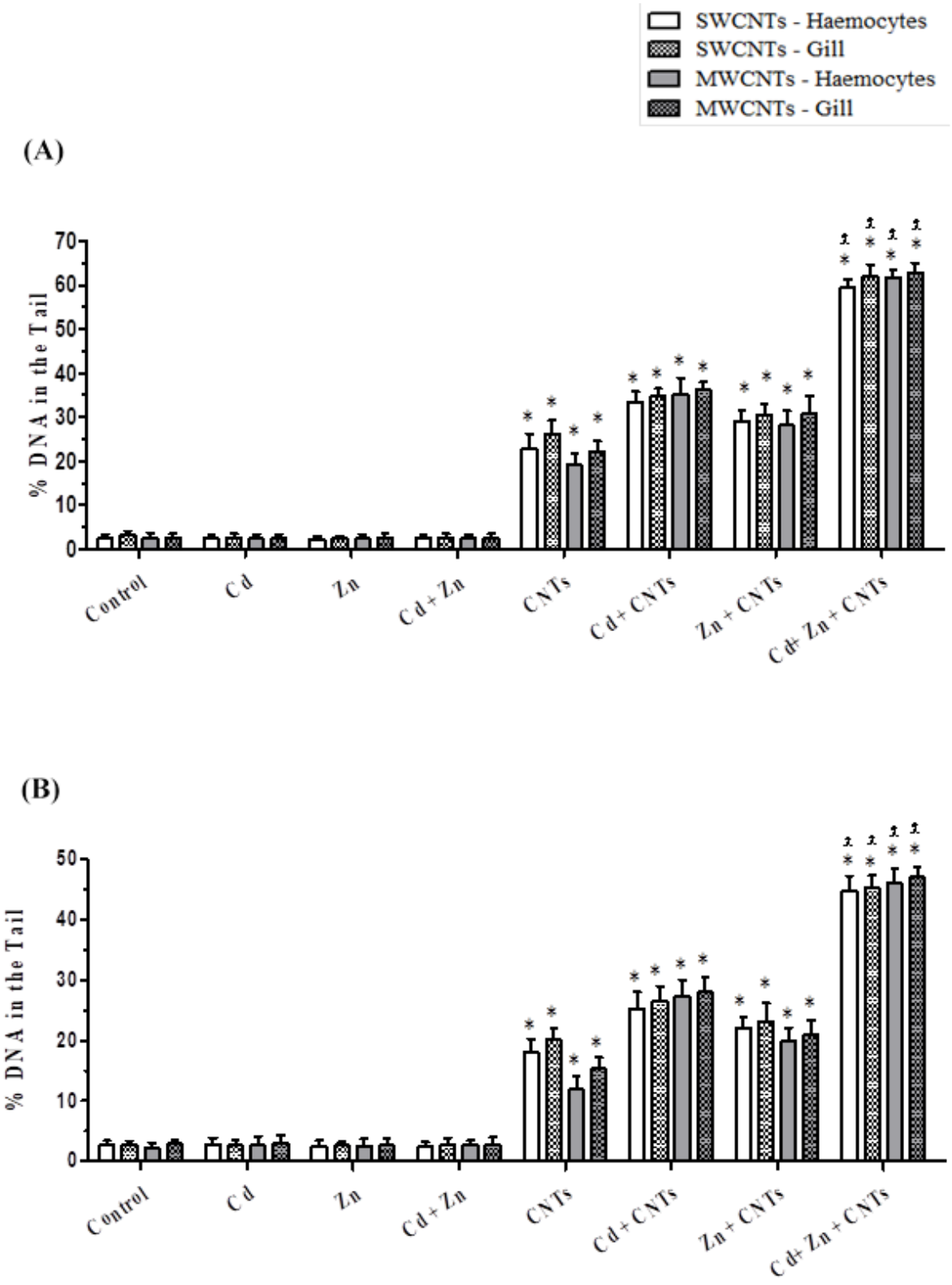


Figure 4.23: DNA damage, expressed as percentage of DNA in the tail, haemocytes and gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at 100µg L^{-1} alone, 100µg L^{-1} + **Cd** 0.001µM, 100µg L^{-1} + **Zn** 1.0µM and 100µg L^{-1} + **Cd** 0.001µM, + **Zn** 1.0µM: in treatment 1 (A) and treatment 2 (B); or equivalent concentrations of CNTs (0.2 $\mu g.g^{1}$) in treatment 3 (C). In all three treatments, statistically significant levels of increased DNA damage were observed in both haemocytes and gill cells. * significantly different from control, Cd, Zn and Cd + Zn; ♣ significantly different from CNTs alone and CNTs + Cd or Zn; ($p<0.05$; means ± standard deviation, n=3).

DNA damage in Cockles (500 µgL^{-1} CNTs)



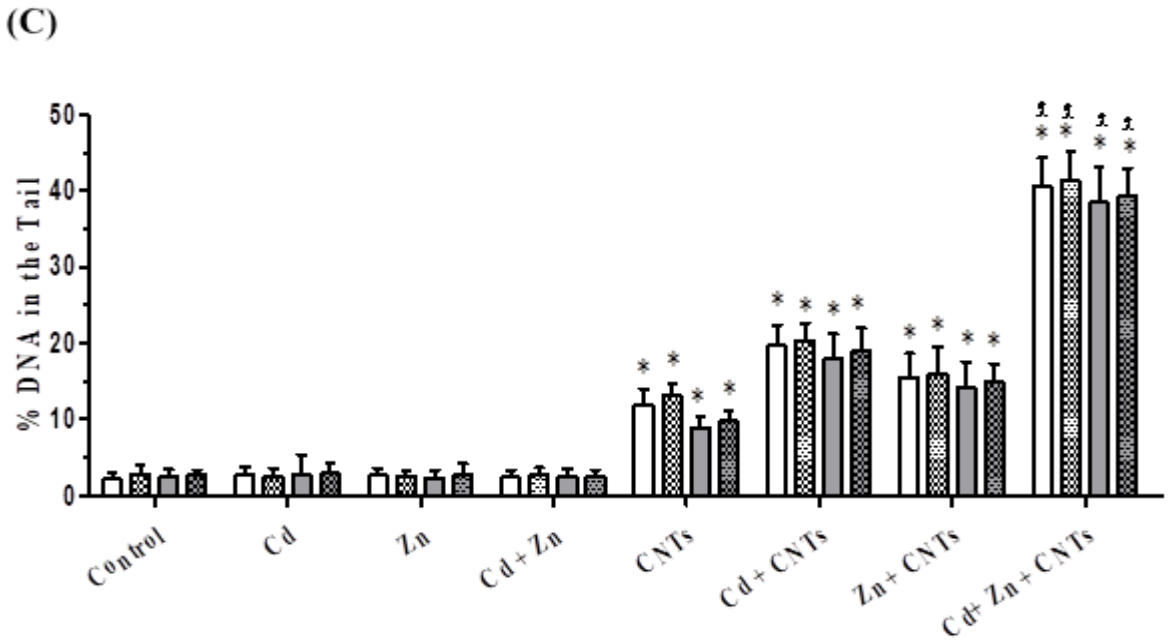


Figure 4.24: DNA damage, expressed as percentage of DNA in the tail, in haemocytes and gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at 500µg L^{-1} alone, 500µg L^{-1} + **Cd** 0.001µM, 500µg L^{-1} + **Zn** 1.0µM and 500µg L^{-1} + **Cd** 0.001µM, + **Zn** 1.0µM: in treatment 1 (A) and treatment 2 (B); or equivalent concentrations of CNTs (1 $\mu g.g^{1}$) in treatment 3 (C). In all three treatments, statistically significant increases in DNA damage were observed in haemocytes and gill cells. * significantly different from control, Cd, Zn and Cd + Zn ; ♣ significantly different from CNTs alone and CNTs + Cd or Zn; ($p<0.05$; means ± standard deviation, n=3).

4.4.5.3 Oxidative stress

This test showed that the exposure of *C. edule* to 0.001µM $CdCl_2$ had no effect on SOD activity (expressed as the percentage of SOD % inhibition) and lipid peroxidation (expressed as TBARS nMol mg protein^{-1}), when compared to the control. Similarlyexposure to either Zn 1µM alone or the mixture of both Cd 0.001µM and 1µM Zn did not increase either SOD activity or lipid peroxidation of *C. edule* in gill tissue (as shown in Figures 4.25, 26, 27, 28, 29 and 30).

However, it can be seen that SOD activity and lipid peroxidation were significantly higher when exposing the cockles to Cd 0.001µM or Zn 1µM, combined with CNTs at concentrations of 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} in treatments 1 and 2 (or at equivalent concentrations 0.01 µg.g^{1}, 0.02 µg.g^{1}, and 1 µg.g^{1} in treatment 3), compared to CNTs alone. Moreover, further increases were observed when CNTs were combined with Cd 0.001µM + Zn 1µM in treatments 1 and 2 or contaminated with the sediment-associated metals in treatment 3 (Figures 4.25– 4.30).

For example, in treatment 3, Figure 4.26C shows that following exposure to a MWCNT concentration of 0.02 µg.g^{1}, the SOD activity (expressed as % inhibition) decreased when exposed to Cd 0.001µM + 0.02 µg.g^{1} MWCNTs (56.2% inhibition), and Zn 1µM + 0.02 µg.g^{1} MWCNTs (62.7% inhibition). However, the SOD activity (expressed as % inhibition) decreased significantly when exposed to Cd 0.001µM + Zn 1µM + 0.02 µg.g^{1} MWCNTs (44.5% inhibition), compared to MWCNTs alone (75.6% inhibition). Meanwhile, for lipid peroxidation (TBARS), the difference

between MWCNTs alone and Cd 0.001µM + Zn 1µM + 0.02 µg.g^{1} MWCNTs was significantly increasd to 4.9 nMol mg protein^{-1}.

There are significant differences in both SOD activity and lipid peroxidation resulting from the exposure of the cockles in treatments 1, 2 and 3 at all studied CNT concentrations. For example, in treatment 1 at 100µg L^{-1} (Figure 4.26A), where the cockle was exposed to Cd 0.001µM + Zn 1µM + MWCNTs, the SOD % inhibition was 19.5%, while in treatment 2 (Figure 4.26B), it was 31.41%, and in treatment 3 (Figure 4.26C), it was 44.58%. This again indicates that treatment 1 leads to higher SOD activity than treatment 2 and treatment 3, which resulted in the lowest SOD activity. Moreover, the TBARS results confirm that treatment 1 resulted in a higher nMol mg protein^{-1} value, while treatment 3 led to the lowest nMol mg protein^{-1}, as shown in Figure 4.26.

Although the SWCNTs alone led to higher levels of SOD activity and lipid peroxidation compared to MWCNTs alone, in all the treatments in this study, it was noticed that, after mixing them with either Cd 0.001µM, Zn 1µM or both metals combined, SWCNTs resulted in levels of SOD activity that were almost equivalent to those produced by MWCNTs. For example, in treatment 3 where the cockle was exposed to 0.02 µg.g^{1} SWCNTs alone, the SOD % inhibition (Figure 4.26 C) was measured at 70.2% inhibition, while when it was exposed to Cd 0.001µM + Zn 1µM + 0.02 µg.g^{1} SWCNTs, it was 43.2% inhibition, showing a significant increase in activity (26.9% inhibition). On the other hand, when the cockle was exposed to 0.02 µg.g^{1} MWCNTs alone, the SOD % inhibition was 75.6%, but this fell to 44.5%

inhibition when exposed to Cd 0.001µM + Zn 1µM + 0.02 µg.g^{1} MWCNTs, a significant increase in activity of 31.0% inhibition. Furthermore, in terms of lipid peroxidation (Figure 4.29 C) under treatment 3 exposure condition, 0.02 µg.g^{1} SWCNTs were found to result in 5.7 nMol mg protein^{-1} and 8.6 nMol mg protein^{-1} for 0.02 µg.g^{1} SWCNTs mixed with metals, with a significant increase in 2.9 nMol mg protein^{-1}. Meanwhile the lipid peroxidation level resulting from 0.02 µg.g^{1} MWCNTs alone was measured as 3.3 nMol mg protein^{-1} compared to 8.2 nMol mg protein^{-1} for 0.02 µg.g^{1} MWCNTs mixed with metals, with a rise of 4.9 nMol mg protein^{-1}.

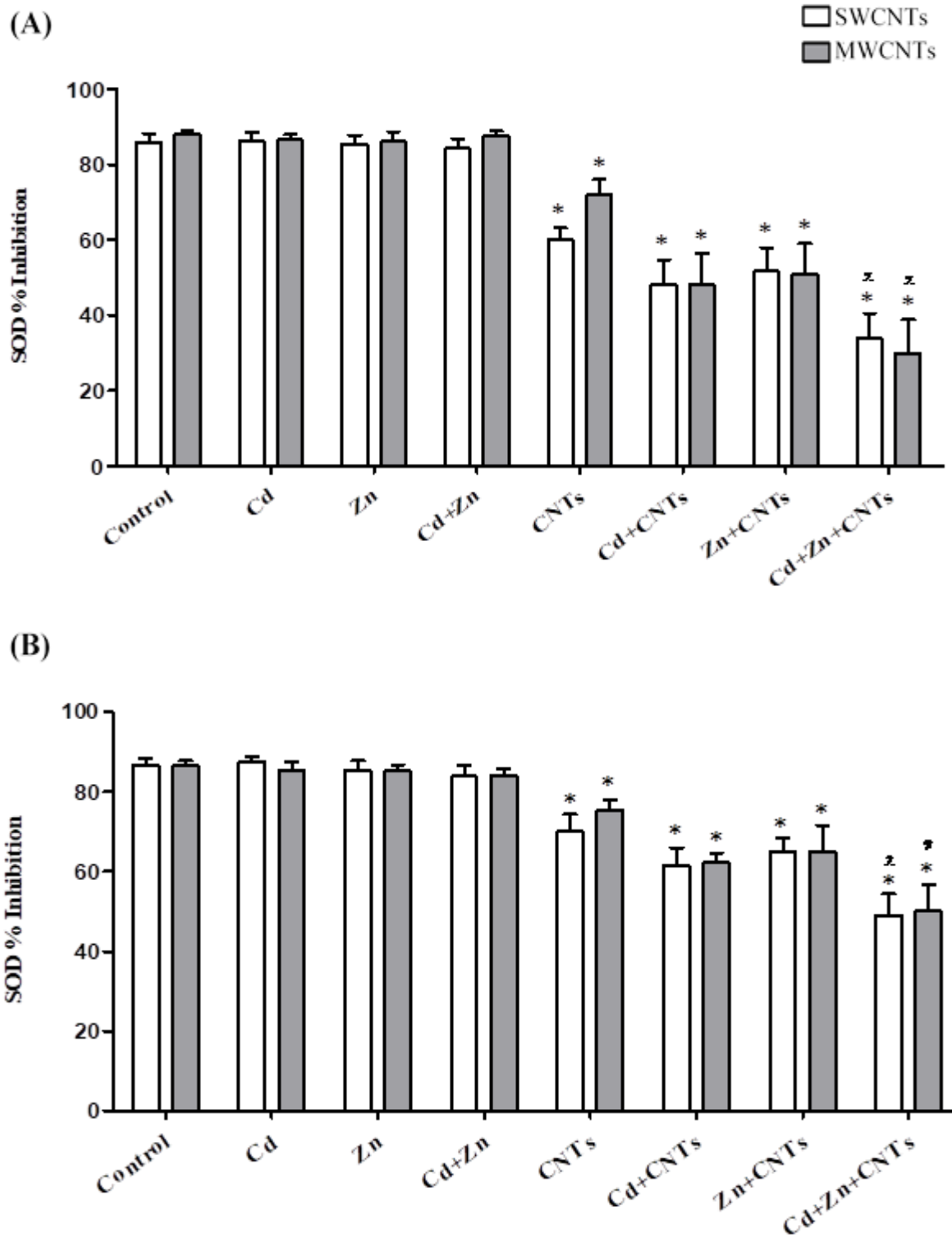
A similar pattern to 0.02 µg.g^{1} was also noticed with exposure to 1 µg.g^{1} of CNTs, where the increased SOD activity and lipid peroxidation only occurred when the SWCNTs and MWCNTs were contaminated with Cd 0.001µM and Zn 1µM, compared concentration of CNTs alone, as shown in Figures 4.27 and 4.30.

Summary

- Increasing SOD activity is an indication of increasing oxidative stress and hence the decrease in SOD % inhibition.
- Increasing concentrations of CNT associated with metals in all treatments resulted in higher SOD activity and lipid peroxidation.
- Metal concentrations of **Cd** 0.001µM, **Zn** 1.0µM and **Cd** 0.001µM + **Zn** 1.0µM had no significant effect on SOD activity and lipid peroxidation when compared to the control.

- SOD activity and lipid peroxidation showed significant increases when SWCNTs and MWCNTs were contaminated with metals, in all treatments.
- SOD activity and lipid peroxidation in cockle cells exposed to MWCNTs increased and became almost equal to the SOD activity and lipid peroxidation in cockle cells exposed to SWCNTs, when the CNTs were exposed to metals.
- The differences in the increases in SOD activity and lipid peroxidation from exposure to MWCNTs were significant than those resulting from exposure to SWCNTs when these CNTs are contaminated with metals.
- For all the studied CNT concentrations contaminated with metals, treatment 1 led to the highest SOD activity and lipid peroxidation compared to treatment 2 and treatment 3, which resulted in the lowest SOD activity and lipid peroxidation.

Activity of superoxide dismutase (SOD) in Cockles (50 µgL^{-1} CNTs)



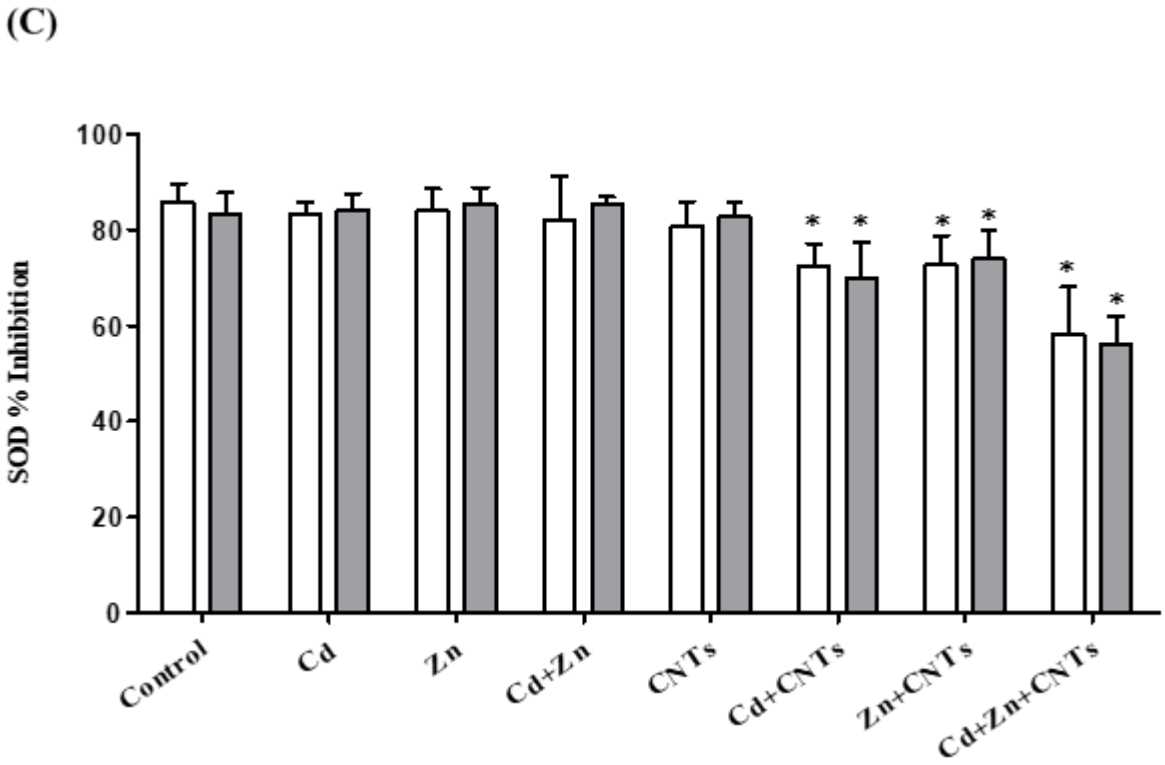
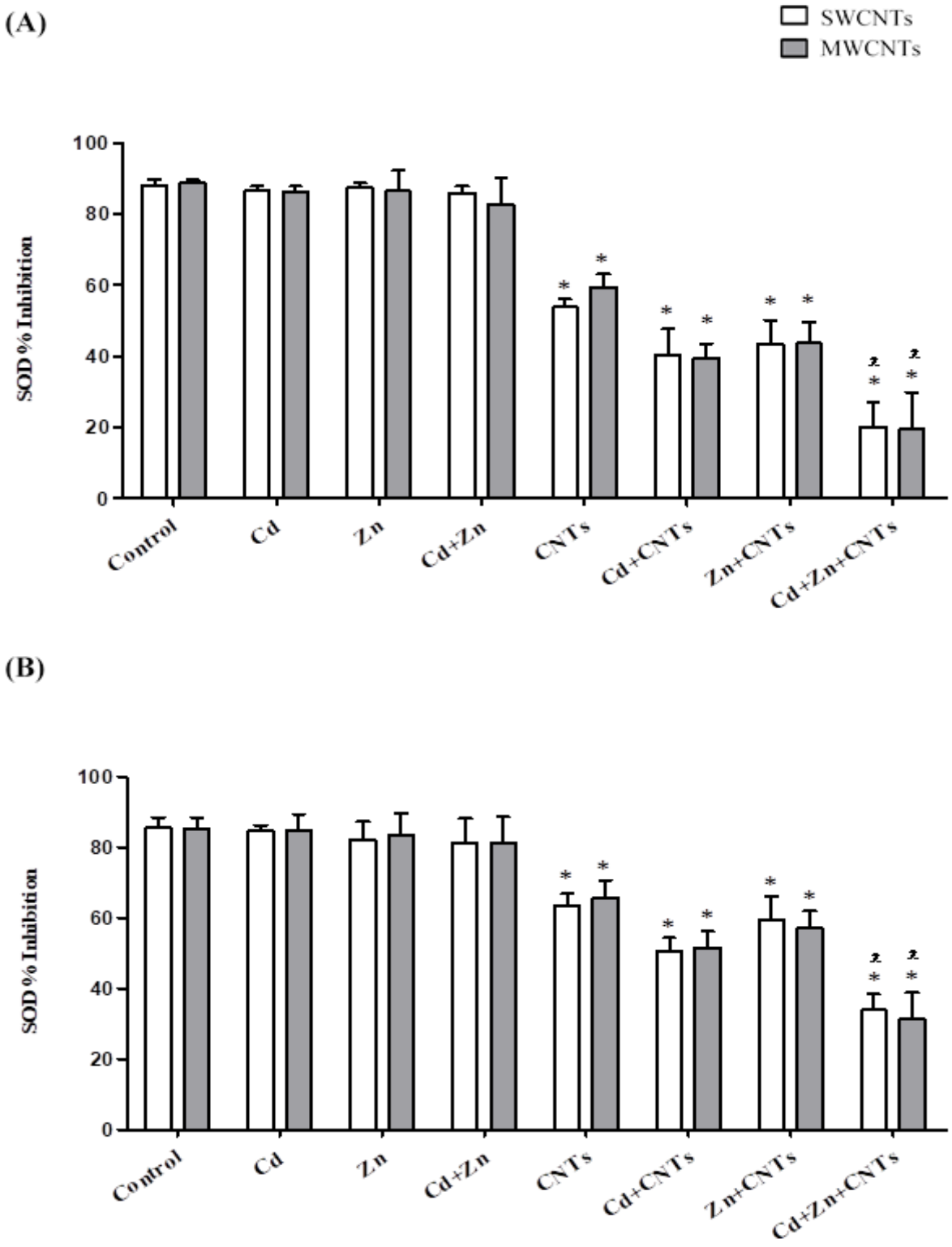


Figure 4.25: Superoxide dismutase activity (expressed as percentage of inhibition) in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at 50µg L^{-1} alone, 50µg L^{-1} + **Cd** 0.001µM, 50µg L^{-1} + **Zn** 1.0µM and 50µg L^{-1} + **Cd** 0.001µM, + **Zn** 1.0µM: In treatment 1 (A) and treatment 2 (B); or equivalent concentrations of CNTs (0.1 µg.g^{-1}) In treatment 3 (C). In all of three treatments, statistically significant increased activity of superoxide dismutase (SOD) was measured in gill cells. * significantly different from control and Cd, Zn and Cd + Zn; ♣ significantly different from CNTs + Cd or Zn; ($p<0.05$; means ± standard deviation, n=3).

Activity of superoxide dismutase (SOD) in Cockles (100 µgL^{-1} CNTs)



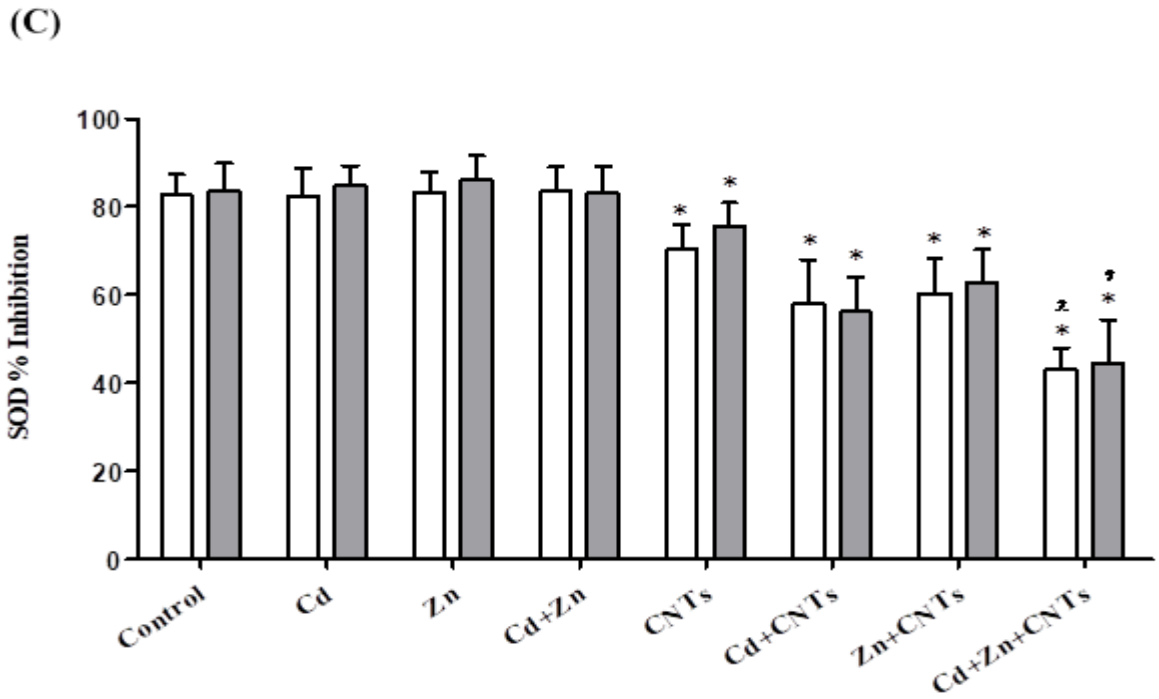
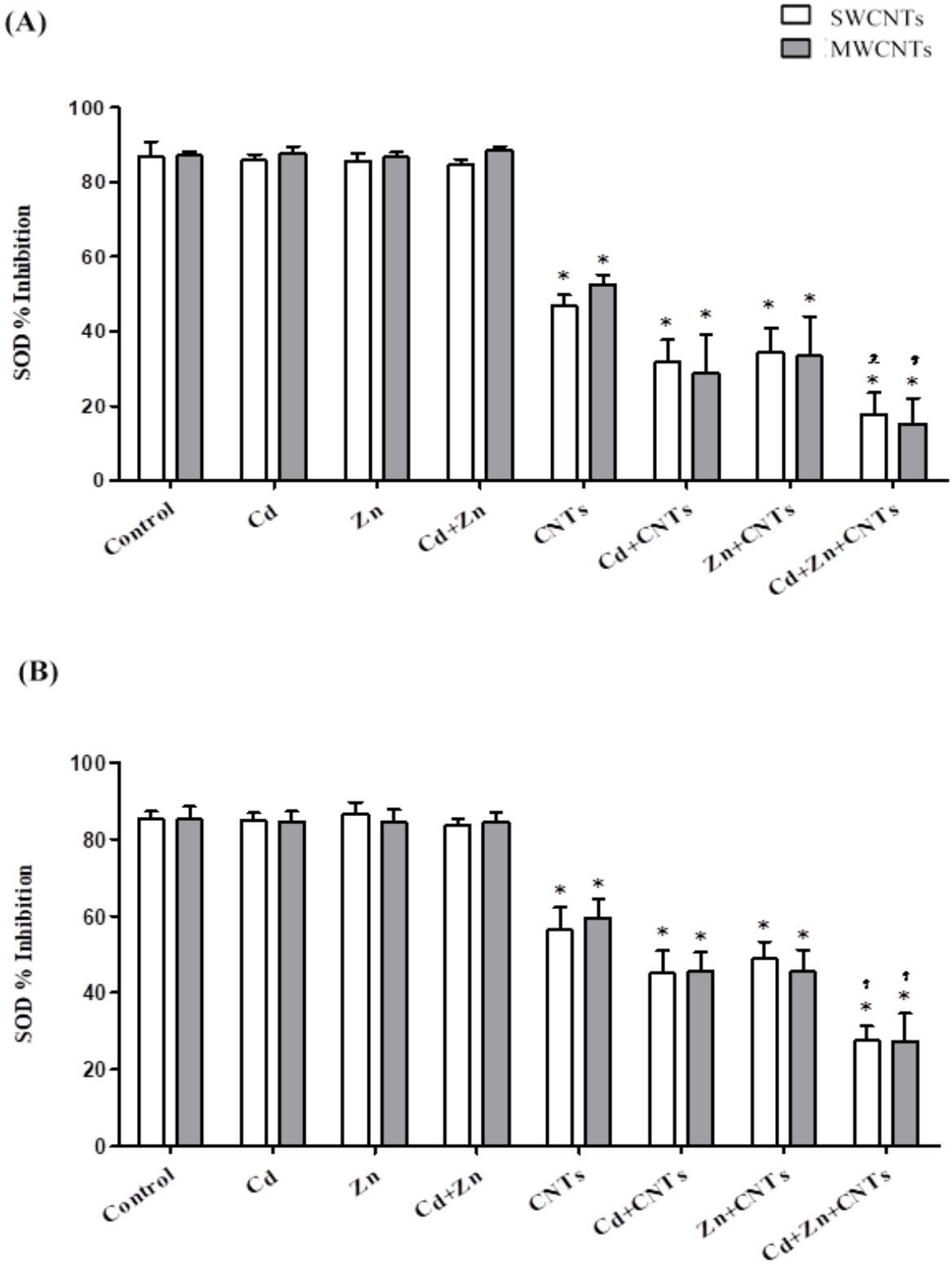


Figure 4.26: Superoxide dismutase activity (expressed as percentage of inhibition) in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at 100µg L^{-1} alone, 100µg L^{-1} + **Cd** 0.001µM, 100µg L^{-1} + **Zn** 1.0µM and 100µg L^{-1} + **Cd** 0.001µM + **Zn** 1.0µM. in treatment 1 (A) and treatment 2 (B); or equivalent concentrations of CNTs (0.2 µg.g^{1}) in treatment 3 (C). In all three treatments. statistically significant increased activity of superoxide dismutase (SOD) was measured in gill cells. * significantly different from control and Cd, Zn and Cd + Zn; ⁂ significantly different from CNTs alone and CNTs + Cd or Zn; ($p<0.05$; means ± standard deviation, n=3).

Activity of superoxide dismutase (SOD) in Cockles (500 µgL^{-1} CNTs)



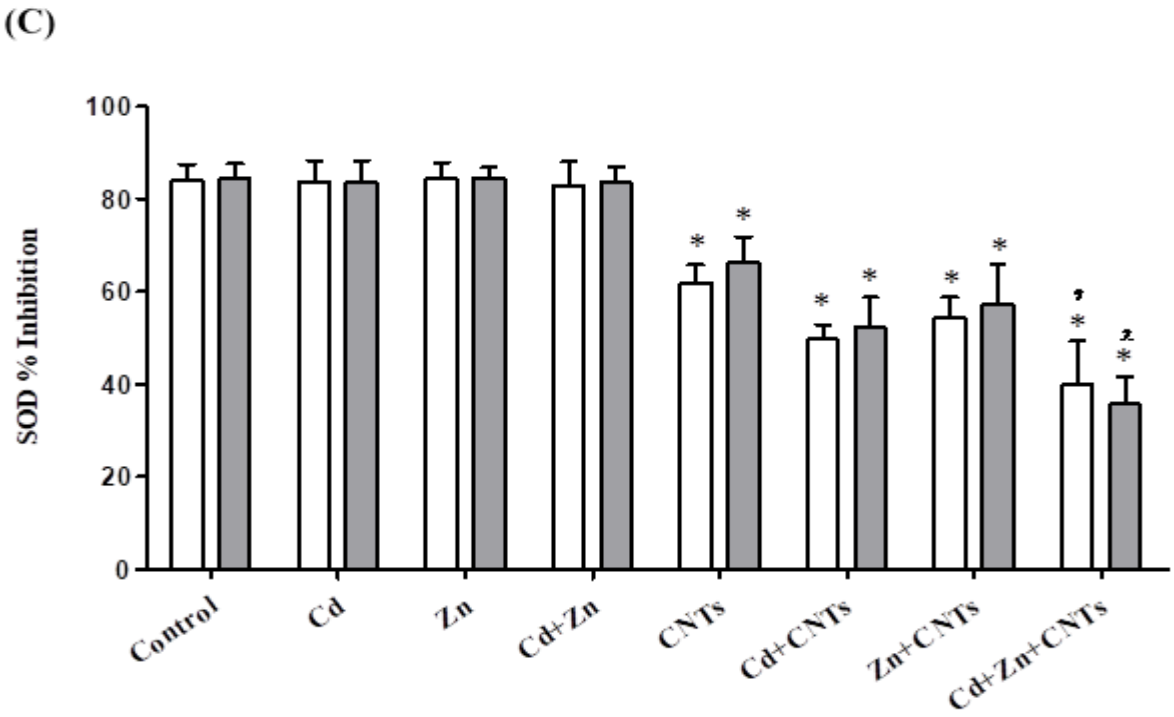
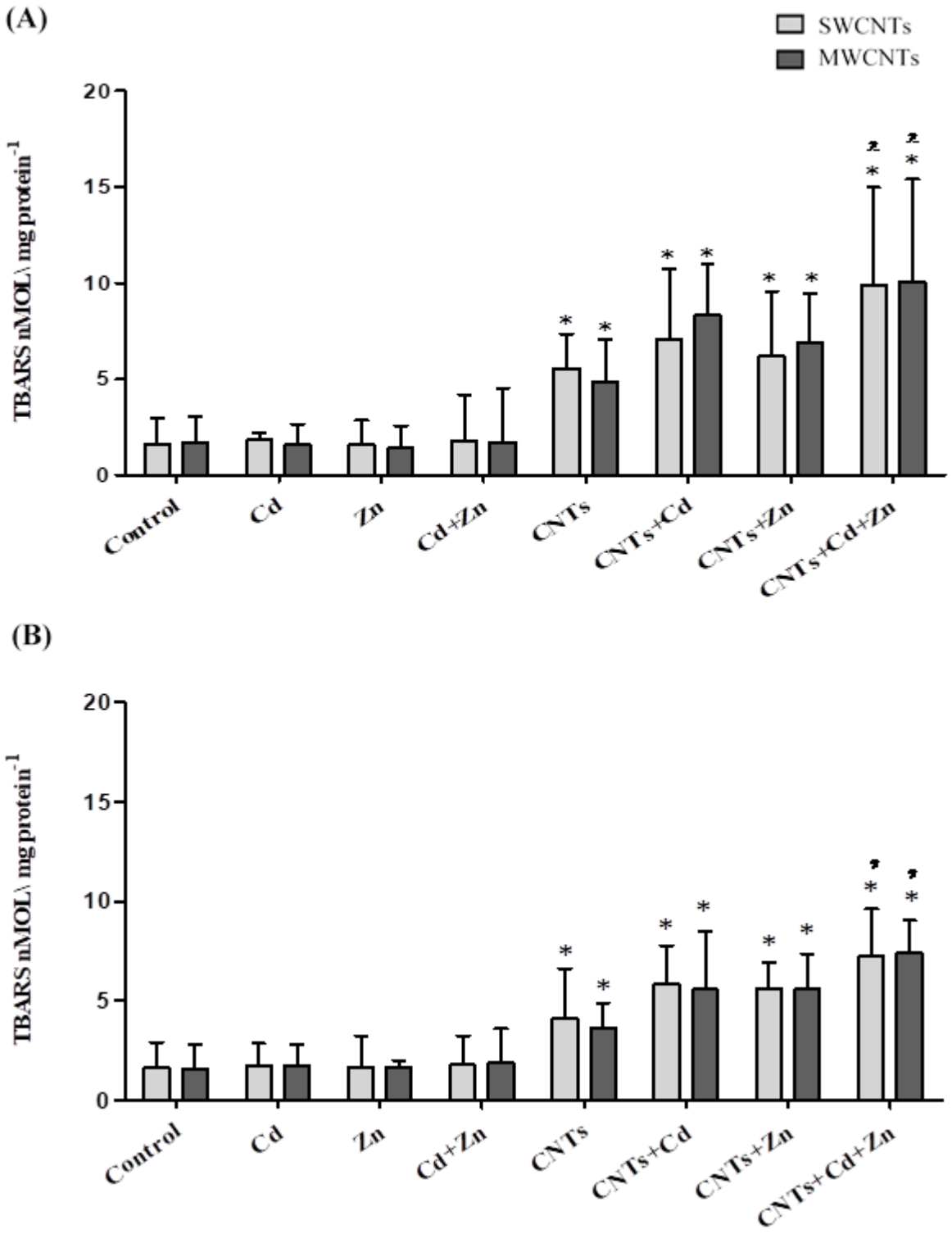


Figure 4.27: Superoxide dismutase activity (expressed as percentage of inhibition) on gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at 500µg L^{-1} alone, 500µg L^{-1} + **Cd** 0.001µM, 500µg L^{-1} + **Zn** 1.0µM and 500µg L^{-1} + **Cd** 0.001µM + **Zn** 1.0µM in treatment 1 (A) and treatment 2 (B); or equivalent concentrations of CNTs (1 $\mu g.g^{1}$) in treatment 3 (C). In all three treatments. statistically significant increased activity of superoxide dismutase (SOD) was measured in gill cells. * significantly different from control and Cd, Zn and Cd + Zn; ♦ significantly different from CNTs alone and CNTs + Cd or Zn; ($p<0.05$; means ± standard deviation, n=3).

Thiobarbituric acid reactive substances (TBARS) in cockles (50 µgL^{-1} CNTs)



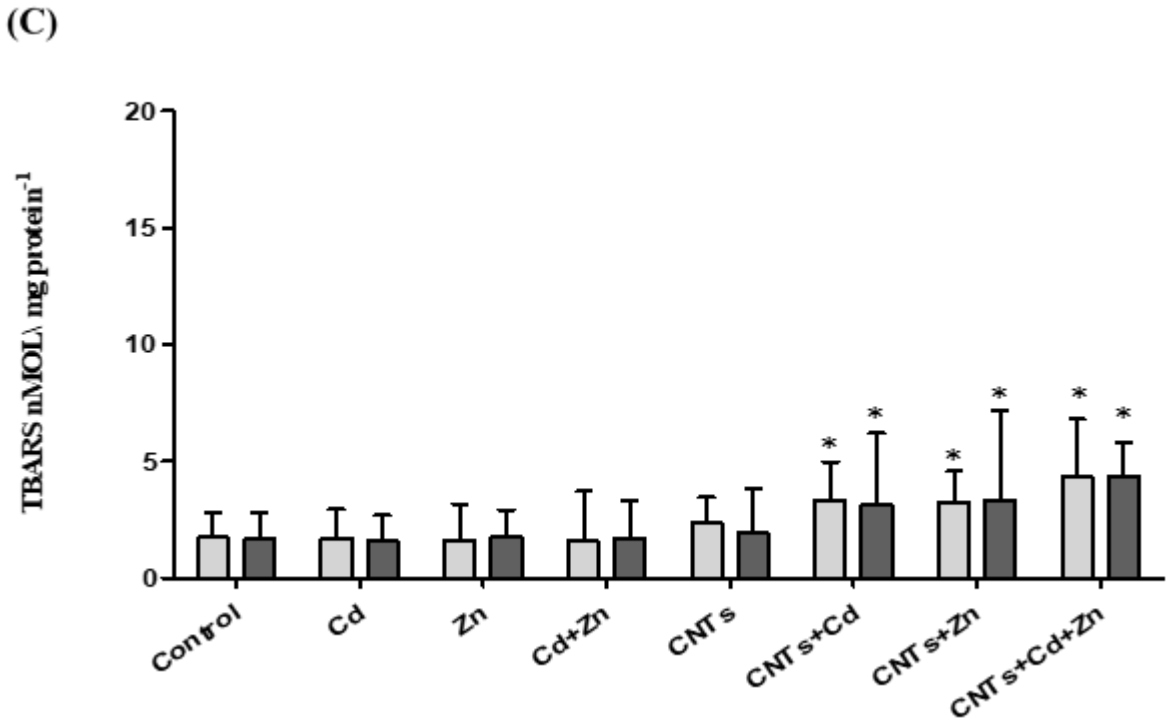
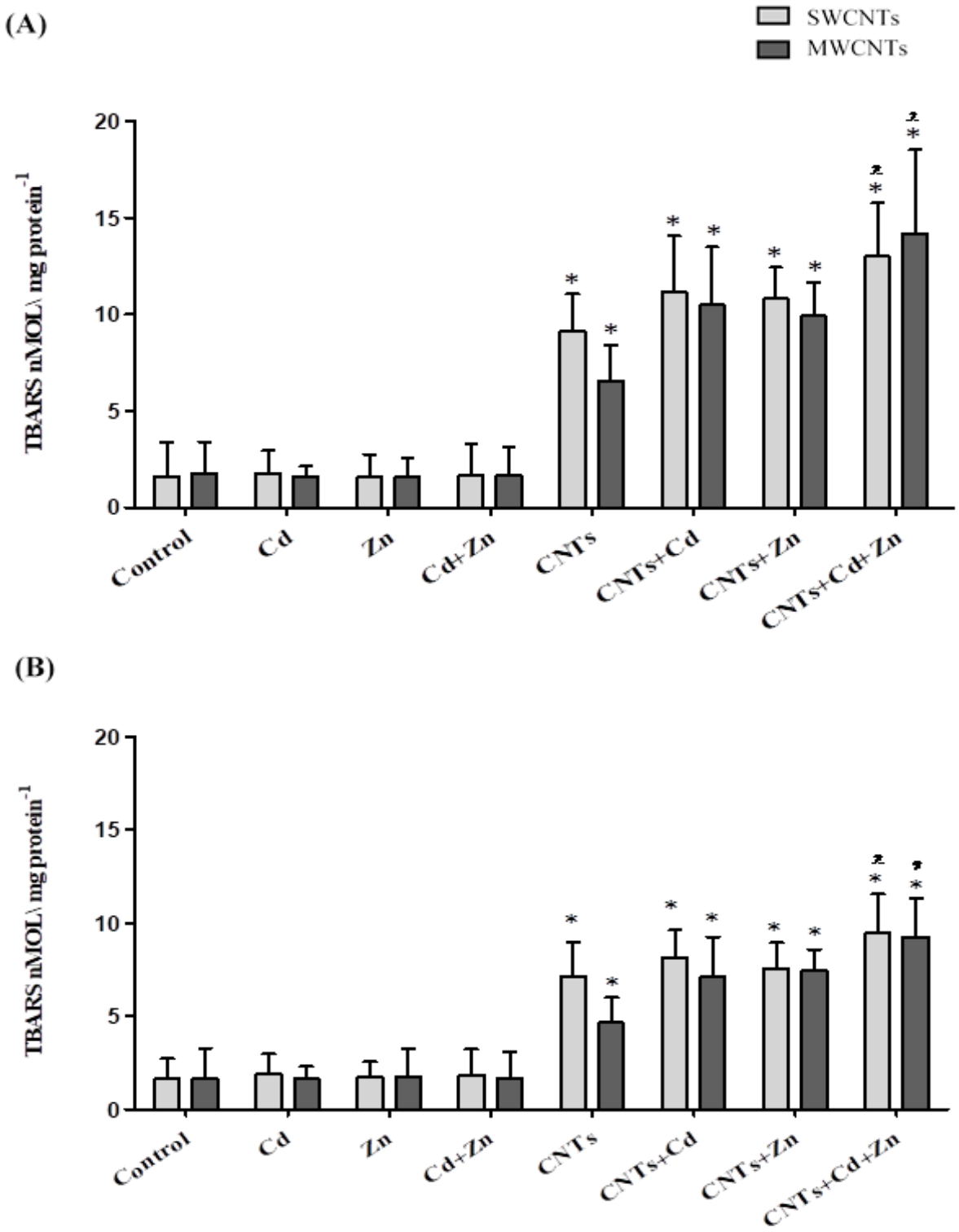


Figure 4.28: Thiobarbituric acid reactive substances (expressed as nMol TBARS per mg protein) in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at 50µg L^{-1} alone, 50µg L^{-1} + **Cd** 0.001µM, 50µg L^{-1} + **Zn** 1.0µM and 50µg L^{-1} + **Cd** 0.001µM + **Zn** 1.0µM: In treatment 1 (A) and treatment 2 (B); or equivalent concentrations of CNTs (0.1 µg.g^{-1}) In treatment 3 (C). In all three treatments statistically significant increased levels of lipid peroxidation were measured in gill cells. * significantly different from control and Cd, Zn and Cd + Zn; ♣ significantly different from CNTs + Cd or Zn; ($p<0.05$; means ± standard deviation, n=3).

Thiobarbituric acid reactive substances (TBARS) in Cockles (100 µgL^{-1} CNTs)



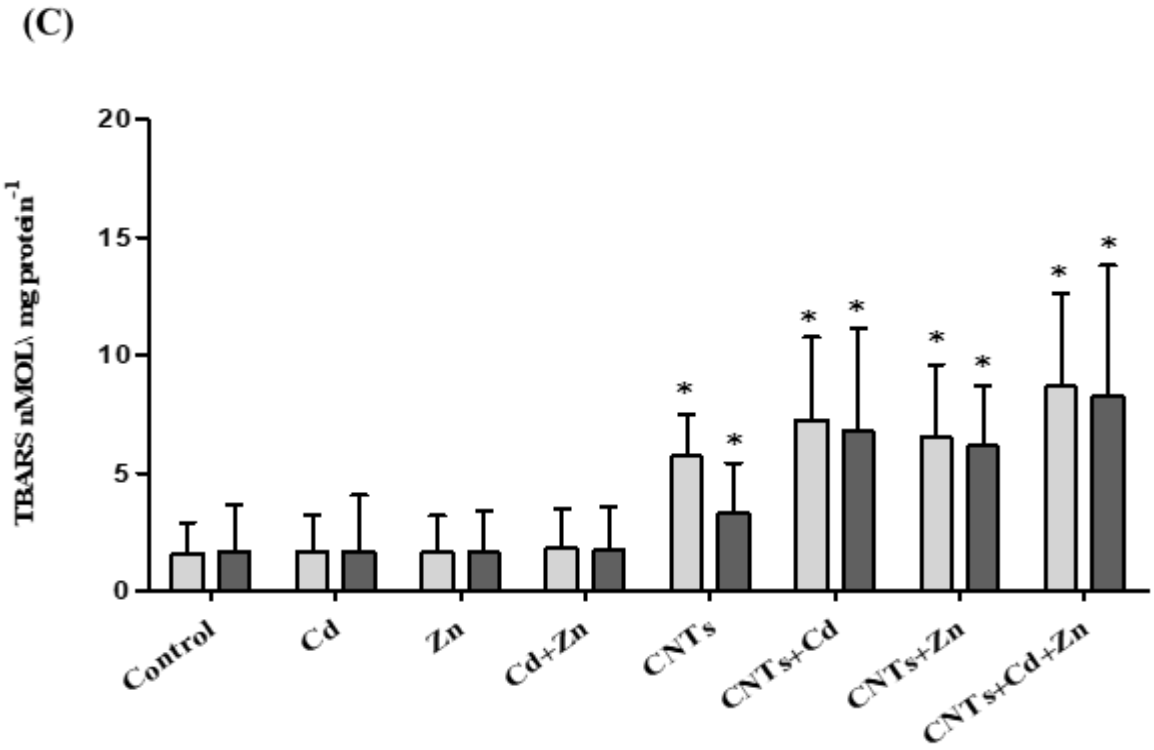
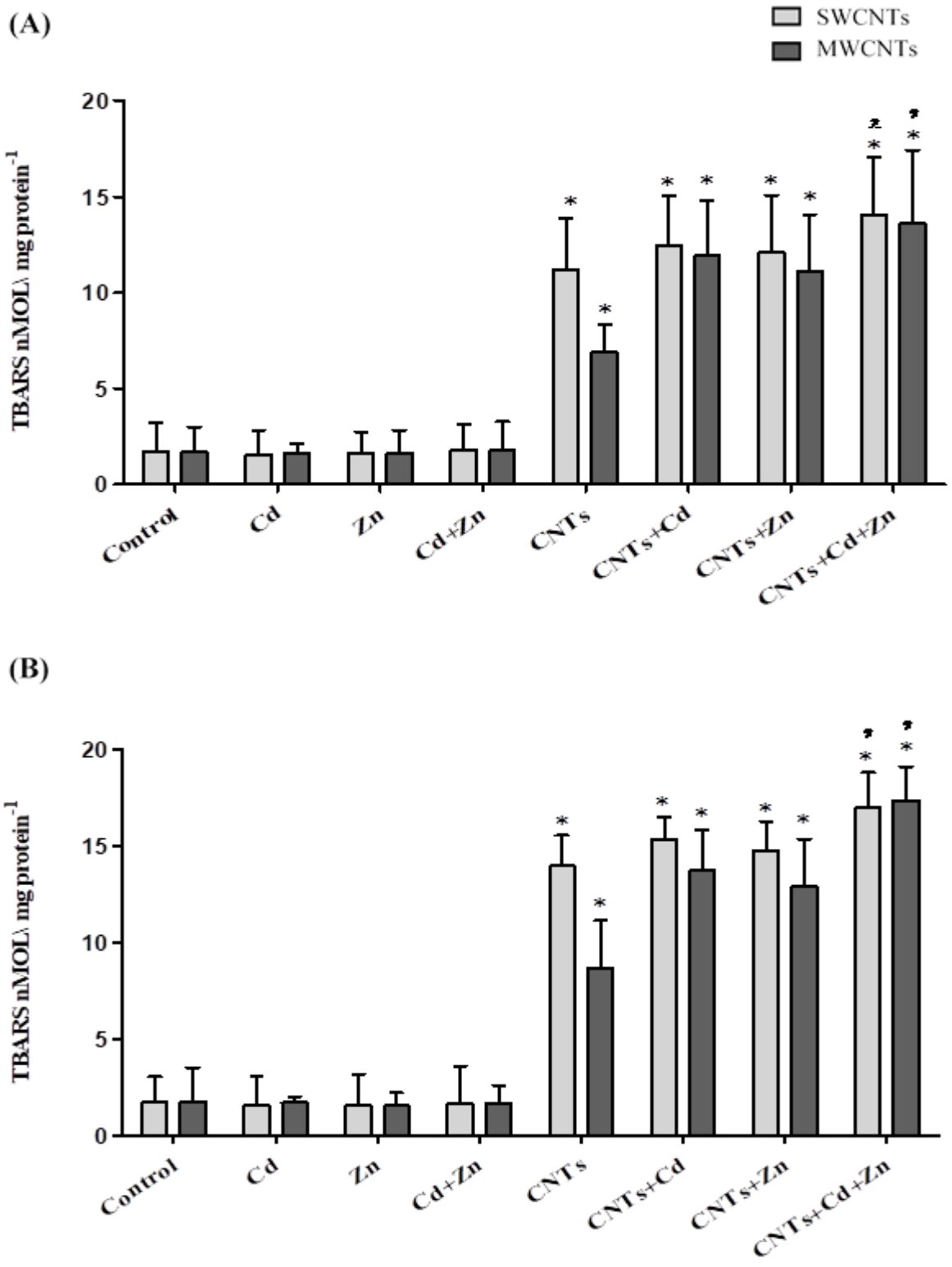


Figure 4.29: Thiobarbituric acid reactive substances (expressed as nMol TBARS per mg protein) in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at 100µgL^{-1} alone, 100µg L^{-1} + **Cd** 0.001µM, 50µg L^{-1} + **Zn** 1.0µM and 100µg L^{-1} + **Cd** 0.001µM + **Zn** 1.0µM: in treatment 1 (A) and treatment 2 (B); or equivalent concentrations of CNTs (0.2 µg.g^{1}) in treatment 3 (C). In all three treatments, statistically significant increased lipid peroxidation was measured in gill cells. * significantly different from control, Cd, Zn and Cd + Zn; ♣ significantly different from CNTs alone and CNT Cd or Zn; ($p<0.05$; means ± standard deviation, n=3).

Thiobarbituric acid reactive substances (TBARS) in Cockles (500 µgL^{-1} CNTs)



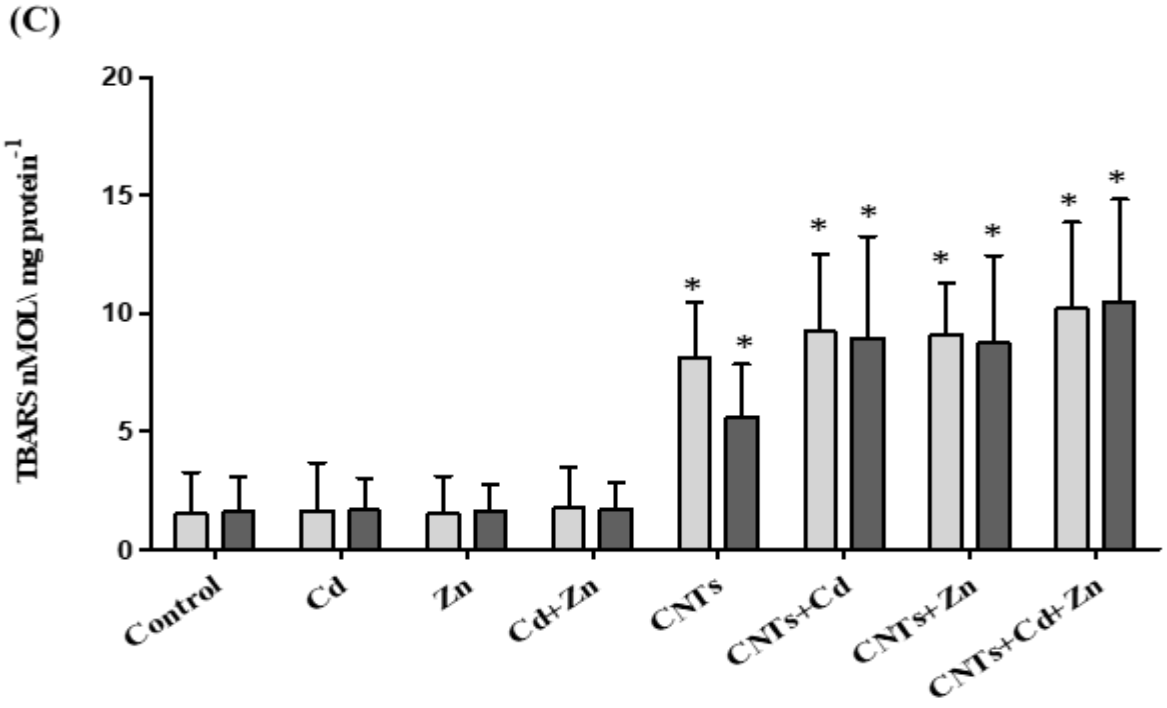


Figure 4.30: Thiobarbituric acid reactive substances (expressed as nMol TBARS per mg protein) in gill cells of *C. edule* exposed *in vivo* to either SWCNTs or MWCNTs at 500µg L^{-1} alone, 500µg L^{-1} + **Cd** 0.001µM, 500µg L^{-1} + **Zn** 1.0µM and 500µg L^{-1} + **Cd** 0.001µM + **Zn** 1.0µM: in treatment 1 (A) and treatment 2 (B); or equivalent concentrations of CNTs (1µg.g^{1}) in treatment 3 (C). In all three treatments statistically significant increased lipid peroxidation was measured in gill cells. * significantly different from control, Cd, Zn and Cd + Zn; ● significantly different from CNTs alone and CNTs + Cd or Zn; ($p<0.05$; means ± standard deviation, n=3).

CHAPTER 5 DISCUSSION

As outlined in previous chapters, there is limited information available on the ecotoxicology of CNTs in the marine environment and, to the author's knowledge, only a few studies have addressed their behaviour in sediments (Petersen *et al.*, 2008; Galloway *et al.*, 2010), where they are expected to accumulate. Understanding the behaviour and environmental fate of CNTs is thus essential to assess their toxicity and risk for the aquatic environment (Hartmann *et al.*, 2014; Stone *et al.*, 2014) and for the identification of particular hazards. To date, the literature has been largely limited to the impact of metallic nanoparticles, with little comparative data obtainable concerning the toxicity of CNTs alone to sediment-dwelling organisms and in the presence of sediment-associated contaminants in the marine environment. In addition, since the toxicity of SWCNTs compared to MWCNTs are not available, this research has been conducted to address some of these knowledge gaps.

5.1 Characterisation of Stock SWCNTs and MWCNTs

Gaining an understanding of the properties of SWCNTs and MWCNTs and how these properties influence their interaction with their environment was an important task of this study. It would allow the understanding of potential bioavailability routes of exposure and toxicity. A variety of methods were applied to carry out the characterisation of MWCNTs and SWCNTs. TEM has been shown to be an effective method for detailed analysis of CNTs and one of the best techniques to characterise

and observe the internal structure of nanotubes, due to its ability to observe the samples in high resolution (Safarova *et al*., 2007). The dispersion and aggregation of SWCNTs and MWCNTs in 0.02% SRNOM as the dispersant was analysed by TEM at Edinburgh University. The TEM micrographs show the morphological structure of the MWCNTs and SWCNTs; they indicate high-purity nanotube samples, with amorphous carbon rarely noted (Figure 4.1). The TEM image shows SWCNTs in thin bundles (Figure 4.1 A-C) and the entanglement of MWCNTs, which provides evidence of the ‘‘bamboo-like’’ structure of MWCNTs (Figure 4.1 D-F) (Branca *et al*., 2004). According to Machado *et al*., (2014), this is a characteristic of MWCNTs, as dispersants causing the MWCNT to behave in this manner. Similar observations were previously described by Jagadish (2015) for MWCNTs and by Al-Shaeri *et al*. (2013) for SWCNTs.

TEM micrographs of the dispersed agglomerates for MWCNTs and SWCNTs prepared in 0.02% SRNOM in seawater are shown in Figure 4.1. The TEM characterisation results showed SWCNTs as a single wall, with less agglomeration (Figure 4.1A-C) than MWCNTs (Figure 4.1 D-F), a reasonable result given the respective structural characteristics of these types of nanotubes. Correspondingly, to keep uncoated CNTs in suspension, dispersants were used during the toxicity experiments. Dispersants such as SRNOM can influence the environmental fate and transport of CNTs and are integral to the bioavailability of materials such as CNTs (Hyung and Kim, 2008). Dispersants can lead to longer exposure times to toxicants for aquatic organisms such as cockles and increase the potential toxic effects. For

example, Smith *et al*. (2007) previously used sodium dodecyl sulphate (SDS) as a dispersant of SWNCTs under exposure experiments in aqueous environments. In several studies, the components of SDS have subsequently been attributed to causing, at least in part, the observed toxic effects (Handy *et al*., 2008; Boyle *et al*., 2014; Petersen *et al*., 2014). As a result, SRNOM, although it is not as good a dispersant as SDS (Alpatova *et al*., 2010), is used because it is considerably less toxic and can still maintain the CNTs suspended in seawater for a sufficient period of time to enable them to settle in an evenly dispersed way on the sediment surface. Furthermore, SRNOM has the most environmental relevance, as it is a naturally occurring organic matter, most likely to resemble a natural compound in an aquatic environment (Bankier, 2012). Therefore, SRNOM was chosen as the best dispersant for use during the experiments with CNTs, and its capability to disperse both types of CNTs and keep them in suspension was confirmed through experimentation.

Despite the natural hydrophobicity of both SWCNTs and MWCNTs, interactions with NOM can stabilise CNTs in aqueous suspension and thus affect their physicochemical fate and biological effects (Hyung *et al*., 2004; Angel *et al*., 2013). Therefore, further instruments were needed to characterise the MWCNTs and SWCNTs' physicochemical properties in seawater. In order to define the approximate size of SWCNT and MWCNT agglomerates, DLS was used (Table 4.1). This is useful to measure the size and the degree of agglomeration or aggregation when testing the nanotoxicological conditions of the material.

To the naked eye, both SWCNTs and MWCNTs prepared in 0.02% with SRNOM appeared to disperse well in seawater. Indeed, although MWCNTs and SWCNTs on their own stayed in suspension for longer than without SRNOM, they eventually became agglomerated, as measurements using DLS proved (see Table 4.1). The surface properties of CNTs, their concentration, and the ionic strength and pH (8) of the medium, as well as their SRNOM-influenced alignment, have also been found to influence their agglomeration behaviour, resulting in reduced electrostatic repulsion and therefore increased homo-aggregation (the aggregate of ENMs with each other) (Bian *et al*., 2001; Zhang *et al*., 2008). A similar behaviour was observed in this study (Table 4.1); however, the level of agglomeration was significantly greater in MWCNTs than in SWCNTs, and this was attributed to an increase in the size-dependent thickness of the diffusion layer of MWCNTs (Borm *et al*., 2006). Moreover, bath sonication or vortex mixing, which is carried out during sample preparation and is commonly used to ensure the dispersion of the ENMs for DLS measurements, can affect the size determination. Consistent with Murdock *et al*. (2008), it was found that the duration of the sonication influenced agglomeration after the initial dispersion.

The level of zeta potential is an important key to stabilise the dispersion collisions. Zeta potential, which can be determined by DLS, is a function of the surface charge of the dispersed particles and it can be interpreted by calculating the magnitude of the mineral surface charge. Zeta potential affects the stability of the agglomerates, as shown in Table 4.1, which shows that MWCNT agglomerates are more stable than SWCNTs, as confirmed by previous studies (Sharma *et at.*, 2014). Zeta potential also

showed that the increased diameter of CNTs affected their charge distribution and dispersion stability in seawater. The present study showed that the SWCNTs and MWCNTs did not have similar zeta potential values (Table 4.1). In general, MWCNTs had a more negative zeta potential charge than SWCNTs, at each studied concentration. In both cases, the CNTs had a more negative zeta potential charge when added to seawater, compared to pure water (Table 4.1).

It has been reported that SRNOM, SDS, and other organic dispersants cause CNTs to align themselves (Lee *et al.,* 2005). Similar patterns were found in the current study, where in the presence of SRNOM, SWCNT and MWCNTs aligned themselves. The results in Table 4.1 show that under stable pH (pH 8) conditions, increases in the SWCNTs and MWCNTs' agglomerate size were dependent on their SRNOM-influenced alignment as well as the CNT concentrations. It has been shown that, in seawater, the surface area and charge of CNTs (as a negative surface charge) are affected by an increase in ionic strength (Joseph *et al.*, 2011). Increased ionic strength induced CNT stability, aggregation and solubility as a result of the less negative zeta potential and the reduced electrostatic repulsion (Wang et al., 2017).

In seawater, the CNTs' physicochemical conditions are likely to have affected the chemical interactions between ionic compounds and CNTs, and therefore influenced their effect on the environment and aquatic sediments. For example, a higher temperature caused faster aggregation due to the increased collision frequency and random Brownian motion, and reduced electrostatic repulsion (Wang et al., 2017).

According to Saito *et al.* (2001) and Machado *et al.* (2013), in the RBM, the G band characteristic peaks have strong Raman intensity in higher (1500-1605 cm^{-1}) and lower (100-450 cm^{-1}) frequency regions, illustrating SWCNTs' sp^2-hybridised bonding nature. In order to detect whether the nanotube is metallic or semi-conducting, the G band uses red laser excitation (785nm) to acquire a signal from semi-conducting nanotubes; otherwise green laser excitement will show that the nanotubes are metallic. The aggregation of CNTs has usually been observed to cause changes in the G band peaks, which may interfere with the identification of metallicity by Raman spectroscopy resonance (Heller *et al*., 2004). As a result of the impact of strong resonance to CNTs' Raman peaks (Kataura *et al*., 1999), implementing a full analysis of all species (n, m) would demand constant laser excitations (785-nm) in order to match every species' (n,m) resonance energies in a CNT (Wanga, 2012). However, the effects of Raman resonance, phonon and the electronic states are mainly dependent on the diameter of the nanotube (Saito *et al*., 2001). As a results of laser energy matching the energy between optically allowed electronic transitions in the CNTs, the observed Raman spectrum, under these resonance conditions, becomes very large, due to the strong coupling that results between the phonons and electrons of CNTs (Dresselhaus *et al*., 2002). Raman spectroscopy resonance is essential to better understand the geometrical structure of the individual CNTs (Dresselhaus *et al*., 2002), and it constitutes a fundamental tool in biological detection (Alivisatos, 2004). For example, Raman detected SWCNT in the kidney, intestine, and bladder of mice in Liu *et al.* (2008), in the gill of *D. magna* in Petersen *et al.* (2013) and in mussels *M. edilus*

(Al-Shaeri *et al.*, 2013). This shows that CNTs have distinctive resonance-enhanced Raman signatures with large scattering cross-sections for single nanotubes, which makes Raman a suitable and sensitive system for identifying SWCNTs (Liu *et al.*, 2009) and MWCNTs (Osswald *et al*., 2007; Bokobza and Zhang, 2012) in biological systems. In this study, for the 1.1 to 1.5 nm diameter SWCNTs produced here, the G band characteristic peak was found to be at a higher (1584 cm^{-1}) frequency, while the RBM was found to be at a lower (268 cm^{-1}) frequency. Moreover, the D band was measured at 1,324 cm^{-1} and the G' band at 2,612 cm^{-1}, as shown in Figure 4.2. While RBM associated with large diameter tubes is too weak to be observed in MWCNTs, Figure 4.3 clearly shows the features associated with the small diameter inner tubes of MWCNTs that were observed in the present study: the G band at ~ 1500–1600 cm^{-1}, the D band at ~ 1,320–1,370 cm^{-1} and the G' band at ~2,682–2,692 cm^{-1}. These results are similar to those of previous studies that showed the characteristic peaks of SWCNTs occurring in the G′-band at 2500–2900 cm^{-1} and in the D band at 1250–1450 cm^{-1} (Dresselhaus *et al*., 2002; Bokobza and Zhang, 2012; Al-Shaeri *et al*., 2013), and as described previously in the same region for MWCNTs (Osswald *et al*., 2007; Bokobza and Zhang, 2012). Supposedly, in conditions of Raman spectroscopy resonance, the Raman intensity empowerment relies on the phonon symmetries of the G band, particularly with regard to SWCNTs (Tang and Albrecht, 1970; Saito *et al*., 2001).

5.2 Bioavailability of SWCNTs and MWCNTs to Sediment-dwelling Cockles

In general, different ENMs display different behaviours when introduced into the marine environment, depending on their chemical composition, mass, particle number, concentration, size distribution, specific surface area, zeta potential, surface contamination, stability and solubility (Klaine *et al*., 2008; Lead *et al*., 2018). At the early stage of spiking (00.00 time) in the *in vitro* test, the CNTs initially dispersed and spread into the whole volume of seawater at the moment of their injection, quickly turning the water turbid and subsequently precipitating onto the surface of the sediment. MWCNTs in suspension were seen to initially agglomerate, float on the surface and subsequently settle onto the sediment. Compared to MWCNTs, SWCNTs settled more slowly, remaining in suspension for at least one hour. Due to the low density of MWCNTs (density at 25ºC: ~2.1 g/ cm^3) (Petersen *et al.,* 2011; Dahlben *et al.,* 2013; Sarma *et al.,* 2015; Ansón-Casaos *et al.,* 2018), it was expected that they would float on the surface for longer and that the subsequent precipitation would occur more slowly than in the case of SWCNTs (density at 25ºC: ~1.7 g/cm^3). Although MWCNTs settle onto the sediment surface more quickly than SWCNTs, the larger agglomerate size of MWCNTs (Table 4.1) made them less bioavailable to cockles, reducing their subsequent toxicity. The slower settlement of SWCNTs onto the sediment surface can be attributed to their better dispersion in sea water and smaller agglomerate size, compared to MWCNTs, and this made SWCNTs more bioavailable to cockles than MWCNTs, increasing their subsequent toxicity (Figure 4.4). Despite

the natural hydrophobicity of both SWCNTs and MWCNTs, interactions with NOM can stabilise CNTs in aqueous suspension and thus affect their physicochemical fate and biological effects, by affecting the surface charge, surface coating and aggregation. Some characteristics may be interconnected, e.g. aggregation, coating and dissolution (Hyung *et al.*, 2007; Angel *et al.*, 2013). CNTs are insoluble in water when added to a simulated marine environment, due to their bundle-shaped configurations (De Marchi *et al*., 2018), high ductility, low density, excellent conductivity, and high mechanical strength (Zhao *et al.*, 2012).

In this study, 2D Raman mapping was successfully used to detect SWCNTs' and MWCNTs' signals in exposed sediments, at a Raman shift of 1,584 cm^{-1} and 1,330 cm^{-1}, respectively (Figure 4.8 and Figure 4.9). 2D Raman mapping has previously been used to confirm the presence of dolomite and cyanobacterial extracellular polymeric substances in complex sediment material and at different depths (Paulo *et al*., 2013). It has been demonstrated that 2D Raman imaging could be further developed as a tool for detecting the presence of CNTs in sediments and other complex media, such as biofilms (Ramya *et al*., 2010).

In this study, the physical interaction between CNTs and cockles was observed by using a light microscope, Raman spectroscopy and TEM. As mentioned earlier, cockle gills are a very effective filter-feeding system that is capable of clearing sub-micrometre sized particulate matter from the water column (Al-Shaeri *et al*., 2013). Consequently, the size of SWCNT and MWCNT agglomerates formed in this study (Table 4.1) was readily taken up by the cockles' gill epithelium in filter feeding. Filter-

feeding cockles react differently to deposit-feeding sediment-dwelling organisms, such as *Lumbriculus variegatus,* which can readily eliminate CNTs (Ferguson *et al.,* 2008; Petersen *et al.,* 2008; Galloway *et al.,* 2010; Petersen *et al.,* 2010; Petersen *et al.,* 2011), albeit only in the presence of food (Koelmans *et al.,* 2009; Petersen *et al.,* 2009; Petersen *et al.,* 2011). Aquatic organisms typically take up CNTs via ingestion, followed by an immediate excretion, and just a small amount of CNTs is detected in the internal organs or blood (Freixa *et al.,* 2018). In this context, 'taking up' means that they enter the mantel cavity, which then either excretes them as pseudofaeces (Figures 4.4 D-G) or ingests and accumulates them in tissues (Figure 4.5). Initial uptake into the mantle cavity was confirmed after the cockle was opened and viewed under the dissecting microscope (stereo microscope). An unquantified amount of CNT agglomerates was observed within the cockle's mantel cavity, and it remained attached to the gill epithelium for at least 72 hrs following the initial exposure (E). Furthermore, a large number of CNT agglomerates could be seen in the digestive gland tissue (Figure 4.6 B and C) and the gill tissue (Figure 4.6 E and F) in histological sections under compound light microscopy, which was similar to the observations of Al-Shaeri *et al*. (2013) with other filter-feeders such as mussels. Based on observations made in this study, it is reasonable to conclude that the size of the SWCNT and MWCNT agglomerates impacts their availability for the cockle's digestive gland, confirmed by the cockles' gill epithelium filter-feeding uptake of CNTs in this species.

Using Raman spectroscopy, the black agglomerates found in the mantle cavity were confirmed to be CNTs (Figure 4.7); they clearly showed the characteristic peaks of

SWCNTs, including the RBM, at low frequencies (Figure 4.7A) and of MWCNTs (Figure 4.7B). These findings are consistent with previous observations, which used Raman microspectroscopy to detect SWCNTs in mussels *M.edilus* (Al-Shaeri *et al*., 2013). The vibrational modes observed for Raman scattering in SWCNTs are distinctive features in the spectrum at 150–350 cm^{-1} (the RBM is related to the nanotube's diameter). Similar RBM results have been reported, after Daphnia magna were exposed to 500 µg L^{-1} SWCNT for 20 hrs (Roberts *et al*., 2007). Raman spectroscopy has also been shown to be a sensitive method for detecting MWCNTs (Osswald *et al*., 2007; Bokobza and Zhang, 2012). In this study, the characteristic peaks of SWCNTs and MWCNTs were not detected in unexposed digestive gland and gill tissue (control) (Figure 4.7 A and B). Based on the Raman evidence and other observations made in this study, it is reasonable to confirms cockles' direct interaction with CNTs through their filtration system (Machreki-Ajmi and Hamza-Chaffai, 2008).

While some studies show NMs and NPs by using TEM, there is still a debate over the mechanisms used and the ability of cells to take up NMs and NPs. For example, it has been reported with the use of TEM that Arenicola marina take up aggregations of TiO2 nanoparticles (NP) through their epithelial membranes (Huang *et al*., 2008; Fabrega *et al*., 2009). Moreover, for vertebrates, it has been shown with TEM that Ag NPs pass into Zebrafish embryo *Danio rerio* s through the pores of the chorion (Lee *et al*., 2007). Previously, a vast amount of SWCNTs has been shown to be accumulated on the gill epithelium of mussels *M.edilus,* which is the main interface between the surrounding environment and the organism (Gomes *et al*., 2011; Al-Shaeri *et al*., 2013). This

study's results support the implication that the uptake of NMs may occur by marine bivalves, with most of them being absorbed in the digestive gland. This confirms the results of Hull *et al*. (2013). Cockles tend to be more resilient to the bioaccumulation of many environmental pollutants (Tanabe *et al*., 1987). In this study, CNTs were shown clearly to suround the cockle's mantle cavity after conducting TEM analysis on the digestive gland and gill cells of the cockle. The agglomerates of ≥100µg L^{-1} CNT were seen to surround the cockles' mantle cavity very clearly. These results show that the concentrations of CNTs in the cockle's body would increase once CNTs have accumulated in the mantle, as the absorption of CNTs into cells and tissues is dependent upon their uptake into the mantle cavity. This might consequently affect the biological system; e.g. by increasing susceptibility to disease, which may result in animal death. In a previous study, lipid-coated SWCNTs were found to be toxic for exposed *Daphnia*, as the aggregation and agglomeration of SWCNTs increased with a longer exposure time (96-h), as did the deposition and clumping of CNTs in the organism's intestines (Kim *et al*., 2010). In another study, several methods were used to suspend the CNTs. This showed that higher toxicity in *Ceriodaphnia dubia* was correlated to high degrees of CNT aggregation, suggesting that the toxicity of CNTs was related to a significant degree to the clumping of these CNTs within the gut (Kennedy *et al*., 2009).

The results of the present study demonstrate the presence of SWCNTs and MWCNTs inside the digestive gland cells (Figure 4.10E and Figure 4.10G) and gill cells (Figure 4.10F and Figure 4.10H), respectively. The smaller size of the CNT particles,

SWCNTs are 0.83 nm in diameter size and MWCNTs are 6 – 9 nm in diameter (Table 3.1), and the agglomeration of MWCNTs that occurred in this study (Table 4.1), might prompt the cockle to distinguish the sizes of the particles absorbed on the surface of the sediment for feeding. This would allow cockles to mainly avoided the larger-sized agglomerated MWCNT particles, and would make SWCNTs more accessible than MWCNTs. As mentioned earlier, the gills of cockles constitute a very efficient filter-feeding system (Dabouineau and Ponsero, 2009), and cockles themselves are considered to be an engineer species that physically disturbs the sediment and nephloid layer through bioturbation (Rakotomalala *et al.,* 2015). Moreover, their patterns of behaviour can also affect their exposure to CNTs. Cockles use their siphons to feed from the sediment surface, and this uptake behaviour would affect the bioavailability of CNTs to the cockle's uptake route which, in turn, affects the level of toxicity.

In this study, the TEM images (Figures 4.10 A-H) show a comparison of the control sample cockles' digestive gland cells and gills cells exposed to 100µg L^{-1} of either SWCNTs or MWCNTs. The results show that the morphology of the cockles' cell membranes changed significantly, and these became subject to breakage, which led to the entry of CNTs into the cell. High aspect ratio property allowed CNTs to cross cell membranes efficiently (Pantarotto *et al.,* 2004) and uptake into organism's cells has been described (Mu *et al.,* 2009). Moreover, there are proposed potential mechanisms of CNT uptake into cells. These mechanisms include using either the passive diffusion or an endocytic pathway to penetrate through cell membranes. The passive diffusion of CNTs results in the simple diffusion of CNTs through the cell membrane without

need of energy consumption (Pantarotto *et al.*, 2004; Shi *et al.*, 2011). There are three stages for the passive diffusion of CNTs through the phospholipid bilayer membrane: 1) landing and floating of the CNTs on the membrane surface; 2) penetration through lipid head groups; and 3) sliding through the lipid tails (Kraszewski *et al.*, 2012). On the other hand, in the endocytic mechanism, CNTs are internalised inside vesicles, and then directed to the lysosomes localised in the perinuclear compartment (Shi Kam *et al.*, 2004). In this study, although cell membrane breakage by CNTs was observed in the cells of the cockles, it cannot be entirely ruled out that this is an artefact (Figure 4.10 F and G). Coupling Raman with confocal microscopy would allow a 3D scan through the fresh cockle's cells without the need for potentially damaging the sample, thus enabling a conclusive assessment of CNT internalisation. The observed effect could be attributable to the destructive effects of reactive oxygen species (ROS), due to the fact that membrane integrity is a primary target (Cabiscol *et al.*, 2000). This study's findings suggest that the CNTs in the present study may have been internalised by the cockles and may also have played a role in reducing the cockles' mobility, although the CNT uptake mechanism is currently unknown. There are several probable explanations for the DNA damage to the gills by the CNTs, resulting in damage to the gill epithelial membranes. Cell membrane breakage can impact the respiration and permeability of the cell membranes, and lead to DNA damage (Klaine *et al.*, 2008). CNTs may cause DNA damage due to their small size, which means that they can pass through the nuclear pores and be transported into the nucleus, where they can interact directly with DNA.

5.3 Toxicity of SWCNTs and MWCNTs to Sediment-dwelling Cockles

In this study, both type of CNTs (SWCNTs and MWCNTs) were confirmed to decrease in haemocytes viability of exposed cockles (*Cerastoderma edule*) species to both type of CNTs for 72 hours at nominal concentrations of MWCNT and SWCNT concentrations of 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} in treatments 1 and 2, and equivalent concentration of 0.1 µg.g^{-1}, 0.2 µg.g^{-1} and 1 µg.g^{-1} in treatment 3.

While cell viability in treatment 3 was higher than in treatment 1 or 2, in all treatments the observed decrease in cell viability was concentration dependent, and was significant only at $\geq$100µg L^{-1} and $\geq$ 0.2 µg.g^{-1} (Table 4.3). This study's results suggest that although the concentrations used might not cause mortality among exposed cockle, they might cause DNA damage and oxidative stress within the cockle cells. Previous studies show that exposing human lungs to 100µgL^{-1} CNTs has no observed effect on cell viability (Tejral *et al.,* 2009; Binelli *et al.,* 2009).

Various studies have investigated the cytotoxicity of NMs but the results can be different because they are affected by different factors, such as exposure period, cell types, test sensitivity and the method selected (Chibber *et al.,* 2013). This study's results show that the viability of cells exposed to SWCNTs was much lower than that of cells exposed to MWCNTs. This is probably driven by many factors, including the large surface area of MWCNTs relative to the smaller size of SWCNTs, which allows more SWCNTs particles to interact with the organisms, causing higher reactivity.

As mentioned above, the Trypan blue assay was utilised in this study for assessing cell viability and testing the haemocytes of *C. edule*. However, there are much more powerful techniques such as flow cytometry (FC), which is recommended and preferable. It has several advantages, including the fact that it is less time consuming and can obtain results with the ability to count 100.000 cells per second, hence providing more reliable results (Diaz *et al.*, 2010).

5.4 Using comet assay to measure DNA damage in cells of cockles

In the current study, a comet assay was applied to measure DNA damage in the haemocytes and gill cells of cockles (*C.edule*). SOD activity (expressed as percentage inhibition) and the degree of lipid peroxidation (expressed as TBARS nMol mg protein^{-1}) were used to measure oxidative stress in the gill cells of cockles after exposure to three different concentrations of SWCNTs and MWCNTs dispersed in SRNOM under three different treatments of exposure conditions, for 72 hrs.

The results showed clearly that most cells in the control and SNORM treatments had minimal levels of damage (less than 5%), which is an acceptable background level arising from either man-made or natural stresses in the field or during the comet assay processes. The dispersant control experiments contained SRNOM at the same concentration as that used for the highest CNT exposure. However, no DNA damage (Figures 4.11, 4.12, 4.13), SOD activity (Figures 4.14, 4.15, 4.16) or generation of lipid peroxidation (Figures 4.17, 4.18, 4.19) was observed in the experiments

containing SRNOM above that observed in the negative control in all three treatments. These findings suggest that any toxicity was not caused by SRNOM.

The rapid increase in CNT usage in industrial and customer products means that they are likely to be released into the environment (Lukhele *et al.* 2015). In this study, the concentrations of CNTs were chosen to be environmentally relevant. It has been proposed that the Predicted Environmental Concentrations (PEC) of CNTs are in the range of 0.001μgL^{-1} –1000 μgL^{-1} (De Marchi *et al*., 2018; Yan *et al*., 2019). Also, it has been shown that 500μg L^{-1} SWCNT was a sub-lethal concentration to mussels (Woods *et al*., 2009). Moreover, Al-Shaeri *et al.,* (2013) exposed mussels *Mytilus edulis* to 500μg L^{-1}, 100μg L^{-1}, 50μg L^{-1}, 10μg L^{-1} and 5μg L^{-1} SWCNTs, and mortality, DNA damage or oxidative stress were not apparent in the mussels exposed to SWCNT concentrations ≤50μg L^{-1}. Moreover, in the present author's preliminary experiments with cockles, no clear DNA damage in *C. edule* was observed for MWCNT and SWCNT concentrations below 50 μg L^{-1}, but the cockles died at exposure levels in excess of 500 μgL^{-1}. Accordingly, the present cockles were exposed to MWCNT and SWCNT concentrations of 50μg L^{-1}, 100μg L^{-1} and 500μg L^{-1} in treatments 1 and 2, and sediment concentrations in treatment 3 equivalent to the amounts spiked in treatments 1 and 2: 0.1 μg.g^{-1}, 0.2 μg.g^{-1} and 1 μg.g^{-1} of MWCNTs and SWCNTs. Both forms of CNTs tested had the potential to cause DNA damage in a concentration-dependent manner to both haemocytes and gill cells, although the level of gill cell damage was higher than that of haemocytes (Figures 4.11, 4.12, 4.13). Moreover, SOD activity, (expressed as percentage inhibition; Figures 4:14, 4.15,

4.16), and the generation of lipid peroxidation, (expressed as TBARS nMol mg protein; Figures 4.17, 4.18, 4.19) in cockles' gill cells exposed to dispersed SWCNTs or MWCNTs responded in a CNT- concentration-dependent manner.

NMs might be toxic to unicellular organisms, such as bacteria and protozoa, or other aquatic organisms, such as Daphnia or fish (Zhu *et al.*, 2006). Some studies have reported that CNTs can be ingested by various species, accumulating in the intestines of oligochaete *Lumbriculus variegatus* (Petersen *et al.*, 2008) and mussel, *Villosa iris* (Mwangi *et al.*, 2012). Moreover, it has been widely proposed that oxidative stress is a likely mechanism that accounts for the toxicity observed after exposure to nanomaterials in aquatic media, particularly CNTs (Kagan *et al*., 2006; Pulskamp *et al*., 2007). Oxidative stress, defined as a disturbance of the equilibrium between antioxidant defences and the production of ROS (Valavanidis *et al*., 2006), is one of the most significant topics of interest for environmental toxicology studies, as it can cause damage to the components of cells and tissues within biological systems. ROS such as superoxide anions, hydroxyl radicals, hydrogen peroxide and other oxygen radicals are capable of directly oxidizing DNA, amino acids in proteins and polyunsaturated fatty acids in lipids (Yoshida *et al.*, 2004). Environmental pollutants can cause various toxic effects that arise at the cellular level; these can be induced by ROS and consequently lead to oxidative stress (Livingstone, 2003; Valavanidis *et al.*, 2006). It has been hypothesised that CNT is likely to be toxic to the mouse epidermis, with the toxicity dependent on the formation of free radicals antioxidant defences (Murray *et al*., 2009). Based on many experiments on oxidative stress and ROS as the

main effects in mammals of exposure to CNTs (Pacuari *et al*., 2008), further clarification is required to better understand the mechanisms of toxicity in invertebrate species (Unfried *et al*., 2007; Mortimer *et al*., 2010; Gomes *et al*., 2011). SWCNT concentrations ≥150µg L^{-1} have been shown to increase SOD activity and lipid peroxidation and induce DNA damage in the hepatopancreas cells of mussels (*Mytilus galloprovincialis*) and snails (*Lymnea luteola*) (Ali *et al*., 2014; Moschin *et al*., 2014).

In this study, following the exposure to either MWCNT or SWCNTs for 72 hrs, there was a significant increase in DNA strand breaks in both haemocytes and gill cells (ANOVA, $P<0.05$), and significantly increased SOD activity and lipid peroxidation in gills (ANOVA, $P<0.05$), but only at concentrations ≥ 50µg L^{-1} under treatment 1 (water spiked) and at ≥ 100µg L^{-1} under treatment 2 (surface spiked) and ≥ 0.02 $g.g^{-1}$ under treatment 3 (sediment spiked) exposure conditions. The results of this toxicity study demonstrate that the increased DNA damage in cockle haemocytes and gill cells, and SOD activity and lipid peroxidation in cockle gills is caused by reactive oxygen species formation. The SOD activity in gills increased after exposure to these CNT concentrations, showing that these CNTs may have potent redox properties (Moschin *et al.*, 2014), with the capacity to generate ROS. Moreover, it has been reported that CNTs also have an ROS scavenging effect (Fenoglio *et al.*, 2006; Petersen *et al.*, 2013), which may be indicative of the formation of superoxide anions in bivalve gills (Gomes *et al.*, 2011) (Figures 4.14, 15, 16). This study result shows that CNTs produce oxidative stress in cockle gills, as evidenced by the increased lipid peroxidation and stimulated antioxidant defence system. It has been suggested that an increased

generation of ROS in various types of cultured cells is a result of exposure to CNTs (Pulskamp *et al.,* 2007; Shvedova *et al.,* 2008). The results of this study are in line with Al-Shaeri *et al*., (2013) study, which used DNA damage oxidative stress markers in another type of bivalve (mussels). Those researchers found that SWCNTs were only toxic to mussels (*Mytilus edulis*) at concentrations ≥ 100µg L^{-1} and that SWCNT-induced DNA damage was correlated with oxidative stress. It has also been reported that if antioxidant defences are deficient, damage may occur, affecting a variety of tissues (Betteridge, 2000).

This study results suggest that the delivery of CNTs via the water column (treatment 1 and treatment 2) is likely to make them more available to the cockles than mixing them with the sediment (treatment 3). In treatments 3, significant increases in DNA damage and oxidative stress effects occurred at concentrations of 0.2 µg.g^{-1} and above, representing a 4-fold decrease in toxicity. CNTs were less bioavailable in treatment 3 (sediment spiked), as they appeared to be only taken up in small amounts during feeding by the cockles, thus resulting in little toxicity being observed. This study highlights how the toxicity of CNTs is dependent not only on the inherent toxicity of the CNTs themselves, but also on how CNTs partition into the benthos and the route of uptake, which is influenced by biological parameters such as the organism's feeding behaviour. There are many reasons explain the lack of CNT uptake in treatment 3 and in part the lack of DNA damage and oxidative stress in cockles under treatment 3 exposure conditions in the present study. First of all, the feeding behaviour of cockles suggests that SWCNTs and MWCNTs had not been absorbed into the tissues by the

cockles, but rather were associated with sediment matter remaining in the gut. The effect of such biological parameters has been reported with observations carried out in sediments for the larva of *Chironomus dilutes*, which creates a tube of sand around itself, totally preventing surface attachment and direct exposure of TiO2 NPs to the body walls (Li *et al*., 2014). Likewise, in a previous study, although lugworm feeding rates and burrowing behaviour were not affected by exposure to SWCNT-spiked sediment (0.003 g/kg), there was DNA damage observed, as measured by comet assay (Galloway *et al*., 2010). The results of this experiment are consistent with a study where TiO_2 deposited onto the sediment surface resulted in greater uptake and toxicity to invertebrates *Hyalella azteca* and *Chironomus dilutes* than when spiked deeper into sediments (Li *et al*., 2014). This is because these organisms, similarly to cockles, feed primarily at the sediment surface. The findings presented here suggest that CNTs mixed with sediment were less toxic to these organisms than CNTs applied to the sediment surface or water column, reflecting the feeding behaviour of cockles and subsequent bioavailability of CNTs. Another reason is that the sediment layers that a particular organism inhabits play an important role in determining the hazardous effects of CNTs, as they can assist in reducing bioavailability and bioaccumulation, leading to the degradation of hydrophobic organic compounds present in sediments (Petersen *et al*., 2009; Petersen *et al*., 2011). Moreover, other studies observed that CNT sorption to sediment and soil particles were able to hinder absorption and lead to a lack of uptake in organisms (Petersen *et al*., 2011). Furthermore, other studies have observed that there was no appreciable absorption outside of the gastrointestinal (GI)

tract (only a very small fraction ~10^{-8} of the total dose) into the tissues of fruit flies (*Drosophila melanogaster*) when digesting food laced with SWCNTs (10 mg/kg), (Leeuw *et al*., 2007). Another reason is the presence of natural organic matter (NOM) which, under specific conditions, can have a complex effect on dissolved metals, which might affect the bioavailability, route of exposure, and hence, toxicity (Nikinmaa, 2014). Therefore, the dispersant employed may also have contributed to the observed lack of toxicity in treatment 3, as the NOM component may have reduced the CNTs' mobility within the sediment because of the colloidal nature of SRNOM (Cerrillo *et al.,* 2015), which coated the CNTs' surface. Therefore, carbon adhered to the sediment grains and molecules and was not bioavailable or accessible to the cockles. SRNOM has been shown to perform a similar function with a range of other nanomaterials in sediment, affecting their subsequent fate and biological effects (Espinasse *et al*., 2007; Pettibone *et al*., 2008; Yin *et al*., 2015).

5.5 Comparing the toxicity of MWCNTs and SWCNTs

Although both types of CNTs eventually settle, leading to their accumulation in sediments and exposure to cockles as benthic-dwelling organisms, a comparison of both types of CNTs tested indicated that exposure to SWCNTs was more toxic to the cells than exposure to MWCNTs under all three treatment conditions. A statistical comparison was carried out between the levels of DNA damage and oxidative stress in cockles after exposure to each type of CNT. SWCNT had the highest percentage of DNA damage (Figures 4.11, 12, 13) and oxidative stress (Figures 4.14, 15, 16, 17, 18,

19) in *C. edule* compared to MWCNTs, regardless of the type of treatment or concentrations. For example, the DNA damage of cells exposed to 500µgL^{-1} (or equivalent concentration 1 µg.g^{-1} in treatment 3) SWCNTs was 27% and 21%, and 13% in the DNA tail of *C. edule*, in treatments 1, 2 and 3, respectively. In contrast, less DNA damage was found in the gill cells of *C. edule* exposed to 500µgL^{-1} (or equivalent concentration 1 µg.g^{-1} in treatment 3) MWCNTs (22% and 16%; and 10% in the DNA tail of *C. edule* in treatments 1, 2 and 3, respectively (see Figures 4.11, 4.12 and 4.13). These findings were consistent with the cell viability results, reflecting the same pattern of toxicity of both forms of CNTs, and suggest that it is likely that the type of CNT plays a significant role in the uptake process, and subsequent toxicity. Size-dependent effects were also observed in life-cycle moulting in Daphnia following exposure to SWCNTs (Oberdörster *et al*., 2006). Furthermore, several studies of CNTs found that the shorter CNTs are more toxic to aquatic organisms than longer ones, therefore SWCNTs, which are shorter, show more toxicity than MWCNTs (Jia *et al*., 2005; Kang *et al*., 2008; Kang *et al*., 2009; Lawrence *et al*., 2016). The smaller particles are likely to have more toxic effects because of their larger specific surface area and their greater interaction with the organism's membrane (Oberdörster *et al*., 2006; Kang *et al*., 2007; Yang *et al*., 2010; Freixa *et al*., 2018). For example, bacteria exposed to individually dispersed SWCNTs showed more toxic effects than bacteria exposed to aggregated SWCNTs, indicating that their toxicity was related to ENM size (Liu *et al*., 2009).

5.6 The Effect of CNTs on the Bioavailability of Sediment-associated Contamination

CNTs have been extensively employed as excellent adsorbents for the removal of the heavy metals Cd (Li *et al*., 2003; Vuković *et al*., 2010; Al-Khaldi *et al*., 2015) and Zn (Shin *et al*., 2011; Vellaichamy and Palanivelu, 2011) from aqueous solutions. Divalent metals ions, for example Cu^{+2}, Ni^{+2}, Pb^{+2}, Zn^{+2} and Cd^{+2}, may be sequestered by CNTs and hence concentrated on the large surface area of CNTs after being removed from aqueous solutions (Qiao and Aluru, 2003; Rao *et al*., 2007). A pH increase in the pH range of 1-8 increases the adsorption of divalent metal ions (Zn^{+2}) by SWCNTs (Lu and Chiu, 2006), and in the current study the metal scavenging behaviour of SWCNTs and MWCNTs was witnessed for Zn^{+2} and Cd^{+2} under exposure conditions (Tables 4.5 and 4.6). It was observed that the concentration of metals (Zn^{+2} and Cd^{+2}) in the supernatant was lower than that associated with the MWCNTs and SWCNTs in the pellet.

It has been shown that in an aquatic environment, CNTs have the property of attracting metal ions (Lu and Chiu, 2006; Rao *et al*., 2007). This property has been widely employed in developing remediate contaminant technologies (Guerra *et al.,* 2018). However, the process by which this occurs can be complicated by the CNTs' surface properties, and the pH and ionic strength of the medium. Nevertheless, Rao *et al.*, (2007) state that at pH 8, the charge of purified and uncoated CNTs is negative. In the present study, a similar situation (salinity 32%, pH 8.4) was found in the seawater

when using DLS and zeta potential to characterise the CNTs' interaction with the metals Cd^{+2} and Zn^{+2}. The expected inverse relationship between zeta potential and pH was confirmed, with negative zeta potentials under slightly alkaline conditions in seawater (Table 4.4). The results showed that, while the zeta potential still had a negative charge in the presence of metals (Table 4.4), the negativity decreased compared to the zeta potential of CNTs alone (Table 4.1). This decrease might be the result of adding the divalent metal ions Cd^{+2} and Zn^{+2} to the negatively charged CNTs, as they offset the negative charges on the CNTs' surface (Al-Shaeri *et al.,* 2013). Given that MWCNTs have multiple layers, unlike SWCNTs, the partial neutralisation between the divalent metal ions Cd^{+2} and Zn^{+2} and the negatively charged CNTs (Dezfoli *et al.,* 2013) increased the zeta potential level for MWCNTs more than for SWCNTs. Subsequently, greater amounts of these metals might be absorbed by MWCNTs than by SWCNTs; metals cross into cockles and therefore increase the toxicity of MWCNTs. SWCNTs and MWCNTs have the capability to adsorb metals with high adsorption capacity (Mubarak *et al*., 2014; Farghali *et al*., 2017). Subsequently, these metals can be accumulated in cockles' tissues (Yap *et al*., 2011), possibly leading to biological effects such as oxidative stress and DNA damage (Gorell *et al*., 1997; Yap *et al*., 2011). Moreover, the results also showed that there is a decrease in agglomerate size compared to when the agglomerate size was characterised for the CNTs alone. Adding the sediment-associated contaminants (Cd^{+2} and Zn^{+2}) to CNTs decreased the size of the agglomerates, due to the decreased

negativity of the surface, and subsequently they were easily spread and absorbed, entering the cockles' tissues and thus increasing the level of toxicity.

5.7 Interaction between CNTs and metals within the cockles

This chemical interaction between CNTs and metals can be viewed as an essential factor to better understand the behaviour of nanomaterial in the environment and its potential effect on biological systems, especially the occurrence of DNA damage. AAS was used to confirm the presence of metals (Cd^{+2} and Zn^{+2}) on the cockles' gill epithelium. The uptake of Pb, Cr, Zn, Cu, Fe, and Cd in cockles' shells and tissues has been confirmed in various studies (Yap *et al*., 2011; Yunus *et al*., 2014; Halit *et al*., 2018). In this study, the chemical analysis of cockles' gill tissue exposed *in vivo* to Zn^{+2} and Cd^{+2} showed higher tissue burdens compared with the control, indicating the bioavailability of the metals under exposure conditions. Moreover, this study shows the chemical analysis of gill tissue from cockles exposed to SRNOM-dispersed MWCNTs and SWCNTs in the presence of Zn^{+2} and Cd^{+2}. The results of this study suggest that the amount of Cd^{+2} and Zn^{+2} accumulated by the cockles was increased when the metals interacted with both types of CNTs. This is likely to be due to the interaction between metals and CNTs, rather than the metals or CNTs on their own. It is attributable to the fact that cockles can take up these metals and accumulate them through their filtration system (Table 4.7). The CNT surface may play an important role in adsorbing other substances, such as metals, from seawater, so that these substances may combine to cause synergistic, potentiating, or additive effects on

exposed organisms, greater than those of the individual substances. If this behaviour is occurring in the natural aquatic environment, it might have implications for CNTs' role in environmental metal dynamics (Kleiner and Hogan, 2003). CNTs have been employed as an outstanding adsorbent for the removal of Zn (Shin *et al*., 2011; Vellaichamy and Palanivelu, 2011) and Cd (Vuković *et al*., 2010; Al-Khaldi *et al*., 2015) from aqueous solutions. It has been reported that free NMs tend to form agglomerates/aggregates in the environment which may be trapped or precipitated and eliminated out of suspension (Farré *et al.,* 2009). This proposes that CNTs may adsorb contaminants such as metals, and this is likely to increase toxicity to a level at which these metals are more toxic than the CNTs or metals alone.

In the present study, a sub-lethal concentration of Cd, 0.001µM, and Zn 1.0µM, was used for *in vivo* exposure, based on the findings of Al-Shaeri *et al*. (2013). After isolation, decrease was observed in cell viability in cockles' haemocytes or gill cells, and the observed decrease occurred in a concentration-dependent manner after exposing them to a combination of CNTs and sub-threshold concentrations of metals (0.001µM Cd or 1.0 µM Zn) alone, under three different treatment conditions (Table 4.8, 4.9, 4.10). However, the cell viability when exposed to CNTs and the sub-threshold concentrations of metals (0.001µM Cd or 1.0µM Zn) decreased above that following exposure to CNTs or sediment-associated contaminants alone. The present data indicate that CNTs are able to cross the cell membrane of cockles and, therefore, might have an influence on cell function. Al-Shaeri *et al.* (2013) report that exposing mussels to SWCNTs 100µgL^{-1}, **Cd** 0.001µM, **Cd** 0.001µM + SWCNTs 100µgL^{-1}, **Zn**

1.0µM, **Zn** 1.0µM + SWCNTs 100µgL^{-1} and **Cd** 0.001µM + **Zn** 1.0µM + SWCNTs 100µgL^{-1} for a 72-hour exposure period had no significant effect on cell viability in mussels' haemocytes or gill cells, and cell viability was still between 85 and 95%. However, they reported a significant increase in DNA strand breaks in mussels' gill cells and haemocytes when exposed to sub-threshold concentrations of metals (Cd 0.001µM or Zn 1.0 µM) and ≥100µg L^{-1} SWCNTs, compared to that following exposure to SWCNTs or metals alone. Moreover, no acute toxicity was observed to impact cell viability after incubating human lung and rat macrophage cells with 100µgL^{-1} commercial MWCNTs and SWCNTs for up to 72 hrs, using quartz and carbon black as reference particles, and acid-treated SWCNTs with reduced metal catalyst content: cell viability was still between 70 and 80% (Pulskamp *et al*., 2007). However, they detected a concentration-dependent increase of intracellular ROS with the commercial CNTs in both cell types after particle treatment, whereas incubation with the purified CNTs (acid-treated SWCNTs) had no effect. The results of these previous studies and the present study lead to the conclusion that sediment-associated metal traces combined with CNTs are responsible for biological effects and cause DNA damage and oxidative stress within cockles' cells.

5.8 Comet Assay and Oxidative Stress

There has recently been a large need to generate ecotoxicity data, and the conceivable adverse effects of sediment-associated contamination, such as dissolved metals that might become associated with ENMs, have been perceived as scientifically important

(Farré *et al*., 2009; Freixa *et al*., 2018). In seawater, the toxic effects of nanomaterials have been attributed to the effect of increased ionic strength on their surfaces area and charges, and therefore their subsequent uptake and exposure by aquatic organisms (Xia *et al*., 2008; Parks *et al*., 2013). In this study, DNA damage in cockles' gills and haemocytes was measured using a comet assay. In addition, oxidative stress was measured by assessing the SOD activity (expressed as a decrease in percentage inhibition) and lipid peroxidation (expressed as an increase in TBARS nMol mg protein^{-1}) in the gill cells of *C. edule*. This study's results demonstrated that there was a concentration-dependent increase in DNA damage in the haemocytes and gill cells of *C. edule*, and oxidative stress in the gill cells of *C. edule* exposed *in vivo* to SWCNTs and MWCNTs for 72 hours, at three different concentrations and under three different exposure conditions. The effects of DNA damage in either gill cells or haemocytes, SOD activity (expressed as percentage inhibition) and the generation of lipid peroxidation, (expressed as TBARS nMol mg protein^{-1}) in the gill cells of *C. edule* following exposure to Cd 0.001µM and Zn 1.0 µM separately or combined, in the absence of CNTs, were not significantly increased above the control. The ROS formation process may cause oxidative damage, like genotoxicity (DNA damage) and lipid peroxidation (Gagné *et al.,* 2008). However, the above results demonstrate that there was no increase in lipid peroxidation or SOD activity, indicating little or no oxidative stress. This means that no DNA damage was detected in *C. edule* exposed to metals Zn 1µM and Cd 0.001µM, separately or combined in the absence of CNTs. The exposure of *C. edule* to Cd 0.001µM did not increase DNA damage or oxidative

stress when compared to the control. Even though Zn 1µM is less genotoxic than Cd 0.001µM, its effect was similar to that of Cd 0.001µM alone. Although Cd is a known carcinogen and genotoxicant (Zhang and Xiao, 1998), including in marine species (Hartl *et al*., 2004), Cd at a concentration ≤0.001µM is not genotoxic to cockles. Zn is known to be less genotoxic than Cd (George and Coombs, 1977; Pruksi and Dixon, 2002), which explains the present study's findings, showing no toxicity at concentrations up to 1µM (Figures 4.22–4.30A, B and C). This study's results suggest that the delivery of Cd and Zn at concentrations of Cd 0.001µM and Zn 1.0 µM, combined or separately, through the water column (treatment 1 and 2) or within the sediment (treatment 3) is not likely to be toxic to cockles in the absence of CNTs. The exposure conditions was varied in this study, but it did not influence the toxic effect of these metals at the above concentrations. Similarly, Pruksi and Dixon (2002) reported no significant increases in DNA strand break levels in mussels' gill cells after exposing them to 0.001µM Cd for 4 weeks, followed by a 1-day exposure to 0.02µM $MgCl_2$ or 0.001µM $ZnCl_2$. Moreover, Al-Shaeri *et al*. (2013) reported consistent findings that no DNA damage or oxidative stress effects were observed in *M. edulis* exposed to Cd 0.001µM and Zn 1.0 µM, combined or separately in a seawater column.

DNA damage resulting from exposing the cockles to ≥50µg L^{-1} (or ≥0.1 $\mu g.g^{-1}$ equivalent concentration in treatment 3) dispersed-SWCNTs or MWCNTs combined with Cd 0.001µM and Zn 1µM, separately, was significantly higher in all treatments, when compared to that resulting from exposure to metals alone. The level of damage was further significantly increased when Cd 0.001µM and Zn 1µM were both

combined with the CNTs, when compared to that resulting from exposure to CNTs alone. For example, in treatment 2 (Figure 4.22B), while the level of DNA damage caused by 50µg L^{-1} SWCNTs in the haemocytes of the cockle was 3.8%, it increased to 6.1% and 5.0% when the cockle was exposed to Cd 0.001µM + 50µg L^{-1} SWCNTs or Zn 1µM + 50µg L^{-1} SWCNTs respectively. The DNA damage was further increased to 12.7 % when the cockle was exposed to Cd 0.001µM + Zn 1µM + SWCNTs. An important physiochemical characteristic is surface charge, which is negative for CNTs in seawater (Table 4.1). Consequently, Cd^{+2} and Zn^{+2} were shown in this study to associate with CNTs, causing a significant genotoxic assault (Figures 4.22, 4.23, 4.24 A, B and C). This is remarkable, particularly as Cd^{+2} and Zn^{2+} alone at concentrations as high as 0.001µM $CdCl_2$ and 1 µM $ZnSO_4$, respectively, failed to indicate any increase in DNA strand breaks compared to the control (Figures 4.22, 4.23, 4.24 A, B and C). The association of carbon nanotubes with metals has been demonstrated to cause biological effects, such as decreasing glutathione levels and cell viability in human keratinocyte cells (Shvedova *et al.,* 2003; Pulskamp *et al.,* 2007). CNTs associated with Ni lead to an increased mice mortality rate, while CNTs associated with Ni are more toxic than CNTs associated with quartz (Lam *et al.,* 2004). This study results indicates that the presence of CNTs associated with the sediment associated contaminants which is divalent ions Cd^{+2} and Zn^{+2} is likely increasing DNA damage more than with the individual contaminants alone.

To conclude, there are clear differences in the levels of DNA damage and oxidative stress between the three treatments with exposure to Cd 0.001µM + Zn 1µM + ≥ 50µg

L^{-1} CNTs. However, the results of this study show that the combined effect of cockles' *in vivo* exposure to both types of CNTs and sub-threshold concentrations of metals on the degree of DNA damage in haemocytes and gill cells, the degree of SOD activity and the generation of lipid peroxidation was significantly ($p<0.05$) elevated compared with exposure to the CNTs or respective metals alone, under all three treatments conditions (Figures 4.23, 4.24, 4.25 A, B and C). For example, DNA damage and oxidative stress were assessed under treatment 3 (sediment spiked) exposure condition at equivalent CNT concentrations: 0.1 $\mu g.g^{-1}$, 0.2 $\mu g.g^{-1}$ and 1 $\mu g.g^{-1}$ spiked into the sediment. There were significant difference in the DNA damage in cockles' haemocytes and gills cells (Figure 4.23 C, 24 C, and 25C). Increased oxidative stress levels in cockles' gill cells (Figure 4.26 C, 27 C, 28C, 29C, 30C and 31C) were observed with exposure to Cd 0.001µM + Zn 1µM + $\geq$ 0.1 $\mu g,g^{-1}$ CNTs, compared to the control. These results demonstrate that the increased toxicological response was associated with the CNTs' interaction with the sediment-associated metals rather than with the CNTs or the metals alone. As mentioned earlier, the CNT surface walls play an important role in adsorbing dissolved metal from the water column. When the metals used in this study were combined with the CNTs' walls they caused potentiating effects on the exposed cockles under all three treatments conditions, hence showing a greater toxicity effect than the metals alone. Potentiating in this context means that the effect results of Cd 0.001µM and Zn 1.0 µM did not shown a toxic effect on their own, but when CNT was added to the metals, they became much more toxic.

5.9 Comparison between MWCNTs and SWCNTs with Metals

CNTs' surface of graphite sheets, which contains hexagonal arrays of carbon atoms, has a strong interaction with other atoms or molecules, which makes CNTs a promising adsorbent material and substitute for activated carbon, in many ways (Liang *et al*., 2004). The CNTs' physicochemical characteristic (e.g., agglomeration state, surface charge, surface chemistry, size distribution, surface area and structure) dictate their behaviour (Pérez *et al*., 2009), which in environmental media are still comparatively unrevealed (Stone *et al*., 2014). In this study, interestingly, the DNA damage and oxidative stress caused by the dispersed-MWCNT were almost equal to those caused by dispersed-SWCNTs, when the metals were combined with SWCNT and MWCNT and spiked in water colum (treatment 1 and 2) or contaminated with sediment (treatment 3). This indicates that the differences in DNA damage and oxidative stress when the metals were added to the CNTs are greater from MWCNTs than from SWCNTs, compared with the difference when using CNTs alone. For example, in treatment 3, Figure 4.26C shows that following exposure to a MWCNTs concentration of 0.02 $\mu g.g^{1}$, the SOD activity (expressed as % inhibition) decreased when exposed to Cd 0.001µM + 0.02 $\mu g.g^{1}$ MWCNTs (56.2% inhibition), Zn 1.0 µM + 0.02 $\mu g.g^{1}$ MWCNT (62.7% inhibition). However, the SOD activity (expressed as % inhibition) decreased significantly when exposed to Cd 0.001µM + Zn 1.0 µM + 0.02 $\mu g.g^{1}$ MWCNTs (44.5% inhibition), compared to MWCNTs alone (75.6 % inhibition). Meanwhile, for lipid peroxidation (TBARS), the difference between MWCNTs alone and Cd 0.001µM + Zn 1.0 µM + 0.02 $\mu g.g^{1}$ MWCNTs was

significantly increase of 4.9 nMol mg protein^{-1}. As mentioned earlier, the CNT surface walls play an important role in adsorbing dissolved metal from the water column. Given that MWCNTs have multiple layers, unlike SWCNTs, this means that MWCNTs have the capacity to absorb more metals and other substances than SWCNTs. MWCNTs show better adsorption capacity for dioxin removal than activated carbon (Long *et al*., 2001), and high efficiency for Pb^{+2}, Cd^{+2} and F^{-} removal from aqueous solution, after oxidation treatment with nitric acid (Li *et al*., 2001; Li *et al*., 2002; Li *et al*., 2003). They can also be used as effective adsorbents for the solid-phase extraction of some organic compounds (Cai *et al*., 2003; Li *et al*., 2004). This indicates that, in the presence of CNTs, the delivery of Cd and Zn at concentrations of Cd 0.001µM and Zn 1.0 µM, combined or separately, through the water column (treatment 1 and 2) or within the sediment (treatment 3) is likely to increase DNA damage, SOD activity and lipid peroxidation; however, the damage resulting from MWCNTs is greater than that resulting from SWCNT.

CHAPTER 6 CONCLUSIONS

Although there has been a recent improvement in comprehension of ENMs' fate in aquatic organisms and model environments, there is a considerable deficiency in essential information regarding ENMs' releases, distribution, persistence and transformations, and their bioavailability in complicated media (Sahu, 2009). Furthermore, research into transport, fate, bioaccumulation and ecological impacts is urgently required, using types of ENMs in representative environmental media and environmentally relevant concentrations with a wider range of organisms (Lowry *et al.*, 2010; Al-Shaeri *et al.*, 2013). This study was conducted in response to this call for further research on this subject

One of the most essential considerations prior to conducting this ecotoxicological experimentation was gaining an understanding of the characterisations of SWCNTs and MWCNTs and how these properties interact with the environment (Petersen, 2014; Stone *et al*., 2014). In this research, CNT characterising techniques such as TEM were fundamental for assessing the size distribution of the nanotubes. Dynamic light scattering and zeta potential provided the agglomerated CNTs' size and surface charge data. Additionally, Raman spectroscopy was important for detecting CNTs. There are various methods of dispersal available, and these might enhance or diminish CNTs' toxicity. To disperse CNTs in water, NOM (natural organic matter, such as humic acids) is considered a good agent (Kennedy *et al*., 2009). This study shows that the capability of SRNOM to disperse both MWCNTs and SWCNTs is good and relatively

stable. It also confirms that the presence of NOM can keep CNTs in a more dispersible and stable suspension (Bennett *et al*., 2013). This study showed that MWCNTs have a greater agglomeration size and more negative zeta potential charge than SWCNTs. Additionally, the study confirmed that the surface properties of CNTs, the ionic strength and pH (8) of the medium, and their SRNOM-influenced alignment, were found to influence their behaviour, fate and effect on the environment and aquatic sediments. To this end, understanding CNTs' characterisation, synthesis, behaviour and functionalisation leads to an understanding of their fate and their effect on environmental and human health.

The NMs released from commercial products and their subsequent environmental impacts are not well understood (Benn *et al.*, 2008). However, some potential release pathways for NMs into the aquatic sediments were hypothesised in section 2.5. Various studies have reported aquatic organisms' uptake of CNTs. The results of this study demonstrate that the availability of SWCNTs inside the digestive gland and gill cells is greater than that of MWCNTs. This was attributed to the smaller size of the SWCNT particles and to the greater degree of agglomeration of the MWCNTs. The agglomeration that occurs in this study can help the cockle to distinguish the sizes of the particles absorbed on the surface of the sediment for feeding, where it mainly avoids the larger-sized agglomerated MWCNT particles; this makes SWCNTs more accessible than MWCNTs. Based on the direct microscopic observations, Raman spectroscopy and TEM observations carried out in this study, it is reasonable to suggest that the size of the SWCNT and MWCNT agglomerates impacts availability

in the cockle's digestive gland, confirmed by the cockles' gill epithelium filter-feeding uptake of sediment-associated CNTs. Cockles' direct interaction with CNTs through their filtration system was also confirmed.

The results of the experiments reported in this study support the hypothesis that the bioavailability of both types of CNTs (SWCNTs and MWCNTs) and their subsequent toxicity were governed by the interaction of the CNTs with the sediments. It was observed that the cockles used their siphons to feed from the sediment surface and this study tested whether cockles' uptake behaviour would increase the bioavailability of the CNTs to the cockles. This was tested under the exposure conditions of treatment 1 (water-spiked) and treatment 2 (surface-spiked) compared to the availability of CNTs mixed into the sediment in treatment 3 (sediment-spiked). It was found that the water-spiked and surface-spiked CNTs were more bioavailable to cockles and therefore they showed higher toxicity than the buried CNTs (sediment-spiked). CNTs were not abundantly bioavailable in treatment 3 (sediment-spiked); they appear to not have been profusely taken up during feeding by the cockles, thus resulting in lower toxicity being observed compared to treatments 1 and 2. This study highlights how the toxicity of CNTs is dependent on not only the inherent toxicity of the CNTs themselves, but also on how CNTs partition into the filter-feeding cockles (*Cerastoderma edule*) and the route of uptake, as the CNTs deposited onto the sediment surface resulted in greater uptake and subsequent toxicity than when spiked deeper into the sediment. This is because cockles feed primarily at the sediment surface. It can be concluded that CNTs mixed with sediment were less toxic to *C.edule* than CNTs applied to the sediment

surface or water column, reflecting the fact that the feeding behaviour of cockles influences the biological parameters and subsequent bioavailability of CNTs. This study suggests that surface-spiked and sediment-spiked CNTs are less toxic than water-spiked CNTs. This reflects another biological parameter, which is that the sediment layers and particles assist in reducing CNTs' bioavailability in surface-spiked and sediments-spiked CNTs, as they hinder absorption. This explains the lack of CNT uptake observed in treatment 3 and, in part, the lower levels of oxidative stress and DNA damage observed in the cockles in treatment 3.

However, this study also found that compared to MWCNTs, SWCNTs were generally significantly more toxic, causing oxidative stress and decreasing cell viability in the cockles' gill cells, as well as increasing DNA damage in both the haemocytes and gill cells of the cockles (*C.edule*). The high genotoxicity of the CNTs is influenced primarily by their special features, including their large surface area, which affects the number of CNT particles released into the exposure medium. Additionally, it affects the uptake process and accumulation inside the cockles' exposed cells, and their subsequent toxicity. Thus, the results from this work provide interesting and novel information on the fate, behaviour, bioavailability and effects of CNTs in a marine benthic organism and they have important implications in the field of environmental risk characterisation of CNTs.

This chemical interaction between CNTs and metals can be viewed as essential to improving our understanding of the behaviour of nanomaterials in the environment and their effect on biological systems. This study shows a direct chemical interaction

between CNTs and metals (Zn^{+2} and Cd^{+2}) as the uptake of metals by cockles was increased when the metals interacted with CNTs, due to the interaction between metals and CNTs. This is likely to be attributable to the fact that cockles take up these substances from the sediment surface through their filtration system, and it confirms that negatively charged surface CNTs were able to strongly adsorb divalent metal ions (Zn^{+2} and Cd^{+2}) on the sediment surface. The adsorption mechanism seems to rely initially on the chemical interaction between the CNTs' surface functional groups and the divalent metal ions in the environment. In the environment, chemical reactions may illustrate the important risk posed by CNTs.

In the cell viability assay, the metals (Cd^{+2} and Zn^{+2}), separately and in combination with CNTs, decreased the viability of either haemocytes or gill cells under all three treatment exposure conditions. Thus, to conclude, CNTs, even when combined with metals, are able to affect cockles' cell viability.

However, in a genotoxicity assay, different scenarios were observed. CNTs, even at low concentrations, when interacting or associating with sediment-associated metals, CNTs were found to have a significant toxic effect. The DNA damage and oxidative stress resulting from exposing the cockles to $\geq 50 \mu g\ L^{-1}$ in aqueous suspensions of SWCNTs or MWCNTs, or $\geq 0.1\ \mu g.g^{-1}$ mixed in to sediments contaminated with Cd 0.001μM or Zn 1.0 μM were significantly higher in both haemocytes and gill cells in all treatments, when compared to that resulting from exposure to sediment-associated metals or CNTs separately. It can be concluded that low CNT concentrations, when combined with metals in aqueous suspensions or sediment-associated metals, are

harmful to sediment-dwelling organisms; this may also be the case for other contaminants stored in sediments. It can be concluded that the chemical interactions between divalent metal ions and nanotubes play a fundamental role in increasing the toxicity of metals. Metals or sediment-associated metals are being made more toxic by the CNTs in aqueous suspensions and sediments because of the partial neutralisation between the divalent metal ions Cd^{+2} and Zn^{+2}, combined with the negatively charged CNT surface wall. This causes potentiating effects on the exposed cockles, showing the significant toxic effect of the metals in the presence of CNTs. This study concludes that the CNTs' surface charge plays an important role in enhancing CNTs' toxicity when they adsorb metals from the aqueous suspensions or sediment.

Furthermore, while SWCNTs alone were found to be more toxic to exposed cockles than MWCNTs, the DNA damage and oxidative stress in MWCNT dispersion were almost equal to those for SWCNTs, when combined with metals. This means that the metal-induced increase in genotoxicity of MWCNTs was higher than that of SWCNTs, compared to the toxicity of CNTs alone. It can also be concluded that MWCNTs show better adsorption capacity for the removal of dissolved metals and sediment-associated contaminant than SWCNTs. The CNTs' surface wall plays an important role in adsorbing metal from the water and sediment medium. Given that MWCNTs have multiple layers, unlike SWCNTs which are single layered, MWCNTs have the capacity to absorb more metals and additional substances compared to SWCNTs.

6.1 Recommendations for Future Research

The potential environmental effects of CNTs are still of concern, even with the current available knowledge regarding the ecotoxicology of ENPs. Firstly, the vast expansion of the use of CNTs in industrial and commercial production means that these nanotubes are likely to continue to accumulate in marine sediments. Secondly, the CNTs' impact on the environment is hard to assess and predict, due to their unknown behaviour and fate in the environment. Therefore, further research and studies are needed in this field. Future work related to this thesis work may focus on the following:

- The behaviour and fate of CNTs in the marine sediments. This needs to be better understood, as this will provide more explanation about ecotoxicity.
- Further research on CNTs' chemical and physical characteristics.
- The mechanisms through which CNTs are transferred to cockles, as a primary target species, through different sediment layers, and via potential food chain sources such as algae.
- CNTs' ecotoxicity on or in different exposure media.

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